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GENETIC ANALYSES OF ADAPTIVE PROCESSES IN THE *PODARCIS* GENUS

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Doctor by the Universitat de les Illes Balears

Universitat de les Illes Balears

Dra. Cori Ramon Juanpere and Dra. Antònia Picornell Rigo, both professors of the Universitat de les Illes Balears.

DECLARE:

That the thesis titles GENETIC ANALYSES OF ADAPTIVE PROCESSES IN THE *PODARCIS* GENUS, presented by Marta Bassitta Sánchez to obtain a doctoral degree, has been completed under our supervision and meets the requirements to opt for an International Doctorate.

For all intents and purposes, we hereby sign this document.

Palma, 4th November 2020

Dra. Cori Ramon Juanpere

Dra. Antònia Picornell Rigo

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Summary

Wall lizards from the *Podarcis* genus, which have diversified and evolved in the Mediterranean basin, present wide morphological, ecological, and genetic variability. This diversity plays a key role in the maintenance of the evolutionary and adaptative potential of all the *Podarcis* populations and, consequently, in their conservation management.

The main point of this thesis was to investigate adaptive processes in the *Podarcis* genus, examining how evolutionary mechanisms have shaped genomic and phenotypic divergence in different *Podarcis* populations. Specifically, it focuses on the *Podarcis* species complex that inhabit the Iberian Peninsula, especially the southeast (SE) region and the Columbretes archipelago, and on one endemic species from the Balearic Islands (*Podarcis lilfordi*). This objective was addressed through different approaches based on specific genetic markers, ecology, morphology, and/or genome-wide analyses.

Regarding the *Podarcis hispanicus* species complex, a multilocus methodology showed that three different clades presenting an overlapping distribution inhabit the SE region. Phylogenetic relationships and geological history enabled a new species to be defined in this region, *Podarcis galerai* sp. nov. and the nominal taxon (*Podarcis hispanicus sensu stricto*) to be renamed. The latter was identified with Albacete/Murcia lineage since it is situated at the closest point to the restricted-type locality of Monteagudo and presents the morphological traits that are most similar to those indicated for the nominal taxon. The taxonomic status of the third group, *P. hispanicus* (Valencia lineage), could not be fully defined due to the lack of sampling in the global distribution. The evolutionary origin of the *Podarcis* form from the Columbretes archipelago was corroborated to be conspecific with the mainland form *Podarcis liolepis*, specifically from the region of Peñagolosa, (Castellón, Spain). The divergence time between the insular and mainland forms was dated at 1.77 Ma coinciding with a sea level fluctuation period, when the two regions could have been connected. Current results disclose low genetic diversity in insular populations, which seems to have suffered diverse events of expansion and/or decrease in diversity (bottlenecks) throughout their evolutionary history.

The extraordinary intra-specific variability present in the endemic lizard from the Balearic archipelago (*P. lilfordi*) was explored at the genetic, morphological, ecological, and behavioural level. The discordance found between phylogenetic, morphological, and ecological results indicates that the use of Evolutionarily Significant Units (ESUs) as taxonomic classification is better to ensure the evolutionary future of these populations and their consideration in conservation policies. Genome-wide analyses using double digest restriction-site associated DNA sequencing (ddRADseq) made it possible to detect more than 70,000 genome-wide single nucleotide polymorphisms (SNPs) that corroborated the uniqueness of these insular populations and highlighted the combined role of genetic drift and natural selection in driving divergence. Tests of selection identified approximately 2% of loci putatively under selection (outlier loci). Correlation analyses with different environmental variables found predation and human pressure as the most explanatory variables in shaping this adaptive divergence. The genetic base of the phenotypical trait of melanism present in several *P. lilfordi* populations was not found in either genome-wide analyses or MC1R candidate gene expression analyses.

Resumen

Las lagartijas del género *Podarcis*, que han divergido y evolucionado en la cuenca Mediterránea, presentan una amplia variabilidad morfológica, ecológica y genética. Esta diversidad juega un papel clave en el mantenimiento del potencial evolutivo y adaptativo de todas las poblaciones de *Podarcis* y consecuentemente, en la gestión de su conservación.

El principal objetivo de esta tesis fue investigar los procesos adaptativos en el género *Podarcis*, examinando como los mecanismos evolutivos han moldeado la divergencia genómica y fenotípica en las diferentes poblaciones de *Podarcis*. Específicamente, se centró en el complejo de especies *Podarcis* que habita la Península Ibérica, en concreto la región sureste (SE) y el archipiélago de Columbretes, así como una de las especies endémicas de las Islas Baleares (*Podarcis lilfordi*). Este objetivo se ha abordado desde diferentes enfoques basados en marcadores genéticos específicos, ecología, morfología, y/o análisis genómicos.

En cuanto al complejo de especies *Podarcis hispanicus*, la metodología multilocus mostró que en la región SE habitan tres clados diferenciados que presentan una distribución solapada. Las relaciones filogenéticas y la historia geológica permitieron definir una nueva especie en esta región, *Podarcis galerai* sp. nov. y redefinir el taxón nominal (*Podarcis hispanicus sensu stricto*). Este último se identificó con el linaje Albacete/Murcia ya que está situado en el punto más próximo de la localidad tipo de Monteagudo y presenta las características morfológicas más similares a las indicadas para el taxón nominal. El estatus taxonómico del tercer grupo, *P. hispanicus* (linaje Valencia), no se pudo definir totalmente debido a la falta de muestreo de toda su distribución. Se corroboró que el origen evolutivo de las *Podarcis* del archipiélago de Columbretes es el mismo que el de la forma continental *P. liolepis*, específicamente de la región de Peñagolosa (Castellón, España). El tiempo de divergencia entre la forma insular y continental fue datada hace 1.77 Ma, coincidiendo con un período de cambios en el nivel del mar, durante el cual ambas regiones pudieron estar conectadas. Los resultados de este estudio revelan una baja diversidad genética en la población insular, que parece haber sufrido diversos eventos de expansión y/o disminución de la variabilidad (cuellos de botella) a lo largo de su historia evolutiva.

La extraordinaria variabilidad intraespecífica presente en las lagartijas endémicas del archipiélago balear (*P. lilfordi*) fue explorada a nivel genético, morfológico, ecológico y de comportamiento. La discordancia encontrada entre los resultados filogenéticos, morfológicos y ecológicos indicó que el uso de las Unidades Evolutivamente Significativas (UES) para la clasificación taxonómica es mejor para asegurar el futuro evolutivo de estas poblaciones y su consideración en las políticas de conservación. Los análisis genómicos usando secuenciación de DNA asociada a sitios de restricción mediante doble digestión (ddRADseq) hicieron posible la detección de más de 70,000 polimorfismos de nucleótido único (SNPs) genómicos que corroboraron la singularidad de estas poblaciones insulares y destacaron el papel compartido de la deriva genética y la selección natural en el impulso de la divergencia. Los test de selección identificaron aproximadamente un 2% de loci supuestamente bajo selección (loci atípicos). Los análisis de correlación con diferentes variables ambientales encontraron que la depredación y la presión humana son las variables más influyentes en la conformación de esta divergencia adaptativa. La base genética del carácter fenotípico del melanismo manifestado en varias poblaciones de *P. lilfordi* no se encontró ni en los análisis genómicos ni en los análisis de expresión génica del gen candidato MC1R.

Resum

Les sargantanes del gènere *Podarcis*, que s'han diversificat i evolucionat a la conca del Mediterrani, presenten una àmplia variabilitat morfològica, ecològica i genètica. Aquesta diversitat juga un paper clau en el manteniment del potencial evolutiu i adaptatiu de totes les poblacions de *Podarcis* i en conseqüència, en la gestió de la seva conservació.

L'objectiu principal d'aquesta tesi va ser investigar els processos adaptatius en el gènere *Podarcis* examinant com els mecanismes evolutius han modelat la divergència genòmica i fenotípica a les diferents poblacions de *Podarcis*. Específicament, es va centrar en el complex d'espècies *Podarcis* que habita la Península Ibèrica, concretament la regió sud-est (SE) i l'arxipèlag de Columbretes, així com una de les espècies endèmiques de les Illes Balears (*Podarcis lilfordi*). Aquest objectiu s'ha abordat des de diferents enfocaments basats en marcadors genètics específics, ecologia, morfologia i/o anàlisi genòmica.

En quant al complex d'espècies *Podarcis hispanicus*, la metodologia multilocus va mostrar que en la regió SE habiten tres clades diferenciats que presenten una distribució superposada. Les relacions filogenètiques i la història geològica permeteren definir una nova espècie en aquesta regió, *Podarcis galerai* sp. nov i redefinir el tàxon nominal (*Podarcis hispanicus sensu stricto*). Aquest últim es va identificar amb el llinatge Albacete/Murcia ja que es troba situat al punt més pròxim a la localitat tipo de Monteagudo i presenta les característiques morfològiques més similars a les indicades pel tàxon nominal. L'estatus taxonòmic del tercer grup, *P. hispanicus* (llinatge Valencia), no es va poder definir totalment a causa de la manca de mostreig a tota la seva distribució. Es va corroborar que l'origen evolutiu de les *Podarcis* de l'arxipèlag de Columbretes és el mateix que el de la forma continental *P. liolepis*, concretament de la regió de Peñagolosa (Castelló, Espanya). El temps de divergència entre la forma insular i la continental es va datar fa 1.77 Ma, coincidint amb un període de canvis en el nivell del mar, durant el qual ambdues regions van poder estar connectades. Els resultats d'aquest estudi van revelar una baixa diversitat genètica en la població insular, que sembla haver sofert diversos esdeveniments d'expansió i/o disminució de la variabilitat (colls d'ampolla) al llarg de la seva història evolutiva.

L'extraordinària variabilitat intraespecífica que presenten les sargantanes endèmiques de l'arxipèlag balear (*P. lilfordi*) va ser explorada a nivell genètic, morfològic, ecològic i de comportament. La discordança trobada entre els resultats filogenètics, morfològics i ecològics va indicar que l'ús de les Unitats Evolutivament Significatives (UES) per a la classificació taxonòmica és més adequat per assegurar el futur evolutiu d'aquestes poblacions i la seva consideració en les polítiques de conservació. L'anàlisi genòmica emprant seqüenciació de DNA associada a llocs de restricció mitjançant doble digestió (ddRADseq) va fer possible la detecció de més de 70,000 polimorfismes de nucleòtids simples (SNPs) genòmics que van corroborar la singularitat d'aquestes poblacions insulars i van destacar el paper combinat de la deriva genètica i la selecció natural en l'impuls de la divergència. Els tests de selecció van identificar aproximadament un 2% de loci suposadament sota selecció (loci atípics). Les anàlisis de correlació amb diferents variables ambientals van identificar la depredació i la pressió humana com les variables més explicatives en la formació d'aquesta divergència adaptativa. La base genètica del caràcter fenotípic del melanisme manifestat a diverses poblacions de *P. lilfordi* no es va trobar ni a l'anàlisi genòmica ni a les anàlisis d'expressió gènica del gen candidat MC1R.

List of publications

1. **Morphological and genetic diversity of the Balearic lizard, *Podarcis lilfordi* (Günther, 1874): Is it relevant to its conservation?**

   Pérez-Cembranos A; Pérez-Mellado V; Alemany I; Bassitta M; Terrasa B; Picornell A; Castro JA; Brown RP and Ramon C.

   Diversity and Distributions (2020), 26(9), 1122-1141.

2. **Multilocus and morphological analysis of south-eastern Iberian Wall lizards (Squamata, *Podarcis*).**

   Bassitta M; Buades JM; Pérez-Cembranos A; Pérez-Mellado V; Terrasa B; Brown RP; Navarro P; Lluch J; Ortega J; Castro JA; Picornell A and Ramon C.

   Zoologica Scripta (2020), 49(6), 668-683.

3. **Genomic signatures of drift and selection driven by predation and human pressure in an insular lizard.**

   Bassitta M; Brown RP; Pérez-Cembranos A; Pérez-Mellado V; Castro JA; Picornell A and Ramon C.

   Scientific Reports (in revision)

4. **Evolutionary history of wall lizards of the Columbretes archipelago.**

   (In preparation)

List of abbreviations

3D	Three-Dimensional
6-Pgdint7	Phosphogluconate dehydrogenase (PGD) gene, exon 7, intron 7
ABGD	Automatic Barcode Gap Discovery
ACACB	Acetyl-CoA Carboxylase Beta
ACSBG1	Acyl-CoA Synthetase Bubblegum family member 1
ACTB	Actin Beta
ACTH	Adrenocorticotrophic Hormone
ADAM2/9	ADAM Metallopeptidase Domain
ADAMTS17	ADAM Metallopeptidase With Thrombospondin Type 1 Motif 17
ADCY1/2	Adenylate Cyclase
AE buffer	Elution buffer
AEMET	Agencia Estatal de Meteorologia
AFLP	Amplified Fragment Length Polymorphism
AIC	Akaike Information Criterion
AICc	Corrected Akaike Information Criterion
alfaMSH	α-Melanocyte Stimulating Hormone
ANCOVA	Analysis of covariance
ANK1	Ankyrin 1
ANKRD13A	Ankyrin Repeat Domain 13A
ANOVA	Analysis of variance
APOBE28	Apolipoprotein B exon 8
ASDDSV	Average Standard Deviation of Delimitation Support Values
ASIP	Agouti Signalling Protein
ATL/AL buffer	Tissue lysis buffer
AW1/AW2 buffer	Wash buffer 1/2
BAPS	Bayesian Analysis of Population Structure
BEAST	Bayesian Evolutionary Analysis Sampling Trees
beta-fibint7	Beta-fibrinogen intron 7
BI	Bayesian Inference
BIC	Bayesian Information Criterion
BLAST	Basic Local Alignment Search Tool
BLASTn	BLAST nucleotide
bp	Base pairs
BPP	for Bayesian Phylogenetics and Phylogeography
bPTP	Bayesian Poisson Tree Processes
BS	Bootstrap Support
CACNA1G	Calcium Voltage-Gated Channel Subunit Alpha 1G

CAMK1D	Calcium/Calmodulin-Dependent Protein Kinase Type 1D
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary DNA
CLIMAP	Climate: Long range Investigation, Mapping, and Prediction
CLIP1	CAP-Gly domain containing linker protein 1
CNKSR2	Connector Enhancer Of Kinase Suppressor Of Ras 2
COI	Cytochrome Oxidase I
COL5A3	Collagen alpha-3(V) chain
COLGALT1	Collagen beta (1-O) galactosyltransferase 1
CR	Control Region
Ct	Cycle number of threshold value
CV	Cross-Validation
CYTB	Cytochrome b
D	Biotic capacity
DAPC	Discriminant Analysis of Principal Components
DCT	Dopachrome tautomerase
ddRADseq	Double digest RAD sequencing
DEPC	Diethylpyrocarbonate
DFA	Discriminant Function Analysis
DNA	Deoxyribonucleic acid
DNAJC17	J domain-containing protein
DNase	Deoxyribonuclease
DnaSP	DNA Sequence Polymorphism
dNTP	Deoxynucleotide Triphosphates
E	Efficiency
EMD	Earth mover's distance
EN	Endangered
ESAM	Endothelial cell Adhesion Molecule
ESS	Effective Sample Sizes
ESU	Evolutionarily Significant Unit
FFT-NS-i	Iterative refinement method
FGFR1	Fibroblast Growth Factor Receptor 1
FHL1	Four and a half LIM domains protein 1 isoform X4
FTH1	Ferritin Heavy Chain 1
G	Shape parameter of the gamma distribution
GDPD2	Glycerophosphodiester phosphodiesterase Domain Containing 2
GEA	Genome Environment Association
GMYC	Generalized Mixed Yule Coalescent
GO	Gene Ontology

GPC1/4	Glypican 1/4
GRM1	Glutamate Metabotropic Receptor 1
GTR	Generalised Time-Reversible
h	Number of haplotypes
ha	Hectare
Hd	Haplotype diversity
He	Expected heterozygosity
HH	Head Height
hierBAPS	hierarchical Bayesian Analysis of Populations Structure
HKY	Hasegawa-Kishino-Yano
HLL	Hindleg Length
Ho	Observed heterozygosity
HPD	Highest Posterior Density
HS6ST2	Heparan-Sulfate 6-O-Sulfotransferase
HSV	Hue Saturation Value
HW	Head Width
I	proportion of invariable sites
id	Individual
ILS	Incomplete Lineage Sorting
InL	Increase in Log-Likelihood
INTS6L	Integrator complex Subunit 6 Like
ITPR2	Inositol 1,4,5-Trisphosphate Receptor type 2
IUCN	International Union for the Conservation of the Nature
JC69	Jukes & Cantor, 1969
JPEG	Joint Photographic Experts Group
K80	Kimura, 1980
k	Number of differences pairwise
kA	Thousand years
kb	Kilobase
Ma	Million years ago
MAF	Minimum Allele Frequency
MAFFT	Multiple Alignment using Fast Fourier Transform
MAP2	Methionine Aminopeptidase 2
MAP7D3	MAP7 Domain-containing protein 3 isoform X1
m.a.s.l.	Meters above sea level
MBNL3	Muscleblind Like splicing regulator 3
MC1R	Melanocortin 1 Receptor
MCC	Maximum Clade Credibility
MCMC	Markov Chain Monte Carlo

MEGA7	Molecular Evolutionary Genetics Analysis Version 7.0
ML	Maximum Likelihood
mPTP	Multi-rate PTP
mRNA	Messenger RNA
MSC	Messinian Salinity Crisis
MSC	Multispecies Coalescent model
mtDNA	Mitochondrial DNA
MWW	Mann-Whitney-Wilcoxon
my	Million years
MYO7B	Myosin VIIB
MYO18B	Myosin motor domain-containing protein
NADH	Nicotinamide Adenine Dinucleotide Dehydrogenase
NCBI	National Center for Biotechnology Information
NCOA1	Nuclear Receptor Coactivator
ND1/2/4	NADH-ubiquinone oxidoreductase chain 1/2/4
Ne	Effective population size
NEGR1	Neuronal Growth Regulator 1
NGS	Next Generation Sequencing
NJ	Neighbor Joining
NMDS	Non-Metric Multidimensional Scaling
NUMTs	Nuclear Mitochondrial DNA segments
OCA2	Oculocutaneous albinism II
OLFM2	Olfactomedin-like domain-containing protein
o/n	Overnight
PA	Private Alleles
PBX3	Homeobox domain-containing protein
PC	Principal Components
PCA	Principal Components Analysis
PCDH7/17	Protocadherin 7/17
PCR	Polymerase Chain Reaction
PGD	Population Genetics Data
Pi/π	Nucleotide diversity
PL	Pileus Length
PO	Posterior Odds
PopART	Population Analysis with Reticulate Trees
PP	Posterior Probability
RAD	Restriction Site Associated DNA
RADseq	RAD sequencing
RAG1	Recombination Activating Gene 1

RAPD	Random Amplified Polymorphic DNA
RDA	Redundancy Analysis
RE	Restriction Enzyme
RGB	Red Green Blue
rjMCMC	eversible jump Markov Chain Monte Carlo
RLT buffer	RNeasy Lysis buffer
RMSE	Root Mean Squared Error
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal RNA
RT-PCR	Reverse Transcription PCR
RT-qPCR	Reverse Transcription Quantitative PCR
RW1/2 buffer	RNeasy Wash buffer 1/2
S	Segregating Sites
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SE	Southeast
SE	Standard Error
SHROOM4	Shroom Family Member 4
SLC9A6	Solute Carrier Family 9 Member 6
SLC24A5	Solute Carrier Family 24 Member 5
SLC45A2	Solute Carrier Family 45 Member 2
SNPs	Single Nucleotide Polymorphisms
SVL	Snout-Vent Length
TACC1	Transforming Acidic Coiled-Coil containing protein 1
TG	Thyroglobulin
TL	Tail Length
tRNA	transfer RNA
TRP1	Tyrosinase related protein 1
TSC	Tortonian Salinity Crisis
TYR	Tyrosinase
UFBoot	Ultrafast Bootstrap
UV	Ultraviolet
VCF	Variant Call Format
VIF	Variance Inflation Factor
WNT10A	Wnt Family Member 10A
ZNF516/711	Zinc finger protein domain-containing protein

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1

Introduction

1.1. Family Lacertidae: genus *Podarcis*

The family Lacertidae (Oppel, 1811) is widely distributed around Europe, Asia, and Africa (Arnold et al., 2007). Forty-two genera belong to this family and there are over 300 different species that inhabit a large diversity of habitats (Baeckens et al., 2015) whose variability includes subarctic tundra, alpine meadows, Mediterranean maquis, steppe, gravel semi-deserts, monsoonal rainforest, and sandy dune systems in the desert (Arnold, 1989; Harris et al., 1998; Harris et al., 2002). Moreover, these lacertid lizards use different substrates within the wide array of habitats, such as herby vegetation over stony undergrounds or shifting sands (Vitt & Caldwell, 2014).

In the Mediterranean area, we find the genus *Podarcis* (Wagler, 1830) which has evolved and diversified in this region. The genus was not widely accepted until its morphological revalidation in 1973 (Arnold, 1973). It includes 23 well-defined species according to international databases (Uetz et al., 2020) and plays an important ecological role in the Mediterranean ecosystems. The distribution of this genus ranges from central Europe to North Africa and from the Iberian Peninsula to the Crimean Peninsula (Figure 1). The peninsulas of southern Europe and associated archipelagos comprise the highest level of species richness and endemic species (Carretero, 2008). Some of these species are in a vulnerable state or endangered, according to the International Union for the Conservation of the Nature (IUCN), while others are successful colonizers. Phylogenetic relationships and, consequently, taxonomic decisions are difficult due to high intraspecific variability and reduced variability between different species inside this genus (Oliverio et al., 2000; Terrasa et al., 2009a). Phenotypic diversity (morphology, colouration, and body size) among the different *Podarcis* species could be explained by their adaptive response to the great diversity of habitats and substrates.

Figure 1. Distribution of the genus *Podarcis* in the Mediterranean basin. Source: Carretero (2008) with minor modifications.

1.1.1. The Iberian and North African *Podarcis*

The *Podarcis* group that inhabits the Iberian Peninsula and North Africa region, except for *Podarcis muralis* (Laurenti, 1768), form a monophyletic group (Harris & Arnold, 1999; Oliverio et al., 2000) which has been described as the *Podarcis hispanicus* (Steindachner, 1870) complex. Several evolutionary lineages have been identified in this species complex, following numerous molecular, morphological, and ecological studies (e.g., Harris et al., 2002; Harris & Sá-Sousa, 2002; Sá-Sousa & Harris, 2002; Pinho et al., 2006, 2008; Geniez et al., 2007, 2014; Lima et al., 2009; Renoult et al., 2009, 2010; Kaliontzopoulou et al., 2011, 2012). Currently, seven lineages are recognized as full species in this species complex (Uetz et al., 2020): *Podarcis liolepis* (Boulenger, 1905), *Podarcis bocagei* (Lopez-Seoane, 1885), *Podarcis carbonelli* (Perez-Mellado, 1981), *Podarcis vaucheri* (Boulenger, 1905), *Podarcis guadarramae* (Boscá, 1916), *Podarcis virescens* (Geniez et al., 2014), and the nominal taxon *Podarcis hispanicus* (Steindachner, 1870). The latter is a paraphyletic group that could include three different mitochondrial DNA (mtDNA) lineages: Valencia lineage, Galera lineage (Pinho et al., 2006, Geniez et al., 2007, Kaliontzopoulou et al., 2011), and Albacete/Murcia lineage (Kaliontzopoulou et al., 2011).

The taxonomy and classification of this group of species is still under review. The most recent geographical distribution (Caeiro-Dias et al., 2018) (Figure 2) of these species indicates that *P. bocagei* inhabits the northwest Iberian Peninsula and *P. carbonelli* has a fragmented distribution along the western Iberian Peninsula. *Podarcis guadarramae* includes two subspecies, *P.* g. lusitanicus, which inhabits northwest and central Iberia; and *P.* g. *guadarramae*, which occupies the central area of the Peninsula. *P. virescens* is found in central and southwest Iberia overlapping with *P. guadarramae*, although with different ecological affinities (altitude and temperature). *Podarcis vaucheri* inhabits the southern part of the Iberian Peninsula and northern regions of African territories such as Morocco and Algeria. *Podarcis liolepis* occupies the northeast of the Peninsula and southern regions of France. The lizards that inhabit the Columbretes Islands (Castellón, Spain) belong to this species (Harris & Sousa, 2002; Renoult et al., 2010). The paraphyletic group of *P. hispanicus* includes the forms that have not yet been elevated to species rank or assigned to one of the other recognized species. The forms that comprise this group (Galera, Valencia, and Albacete/Murcia lineages) inhabit the southeast (SE) Iberian Peninsula.

The actual phylogeny of this group based on mtDNA alignment of 2,291 bp (corresponding to fragments from 12S rRNA, 16S rRNA, control region, cytochrome b, and ND4) (Kaliontzopoulou et al., 2011) differentiates three groups. On the one hand, i) a group made up of two lineages from eastern Iberia (*P. liolepis* in the northeast and southern France, including the Columbretes Islands, and Galera lineage in the SE) that occupies a basal position in the phylogeny. On the other hand, two sister subclades: ii) one in west and central Iberia (*P. bocagei*, *P. guadarramae*, *P. carbonelli* and *P. virescens*); and iii) the other in SE Iberia and northwest Africa (Valencia lineage, Albacete/Murcia lineage and *P. vaucheri*) (Figure 3). The cluster of western and central forms and the southern and African clade diverged approximately 8.98-11.71 Ma, according to Kaliontzopoulou et al. (2011). This dating coincides with the formation of the cluster (*P. liolepis* and Galera lineage) at 9.15-10.09 Ma. In this period, the Betic marine corridor opened (Figure 4) and the fragmentation of the area which gave rise the Betic region took place (8-10 Ma). This region remained as an archipelago not colonisable by land until the end of the Tortonian (7.2 Ma) when the Betic Strait was closed (Duggen et al., 2003).

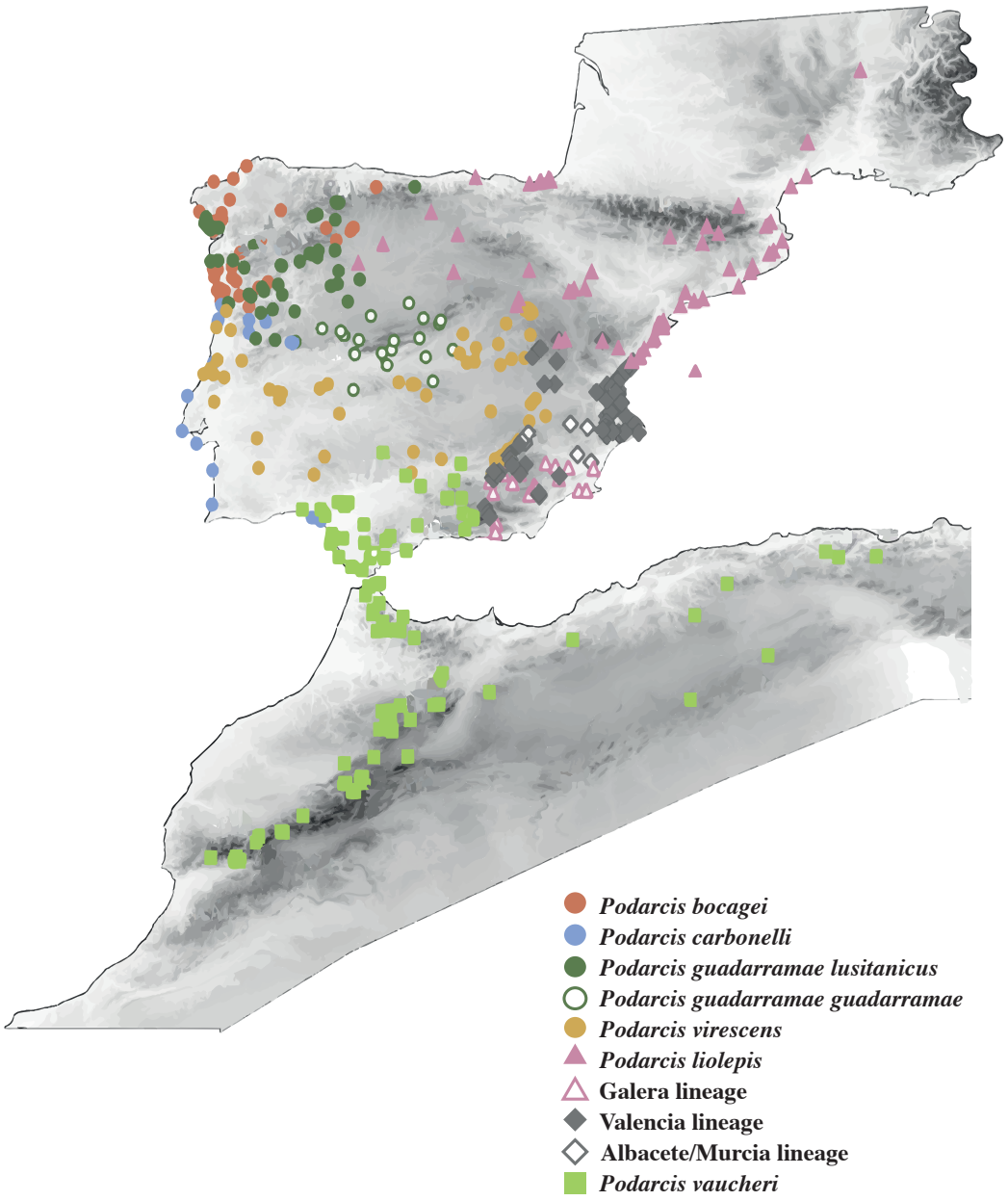


Figure 2. Distribution of main *Podarcis* mtDNA lineages from the Iberian Peninsula and North Africa. Source: Caerio-Dias et al. (2018) with modifications.

The transformation of the Betic marine corridor into a shallow sea level environment led to the Tortonian Salinity Crisis (TSC) (Krijgsman et al., 2000) which caused the formation of land bridges between the Peninsula and islets in what is now the Betic region. These areas may have been colonized by lizards and become a speciation hotspot (Paulo et al., 2001). The final closing of the Betic Strait was responsible for the Messinian Salinity Crisis (MSC) (5.6-5.3 Ma) causing the drying of the Mediterranean Sea, allowing for the connection between the continents, and enabling the possible colonisation of these lizards to North Africa. The opening of the Strait of Gibraltar at the end of the MSC (5.3 Ma) caused the rapid refilling of the Mediterranean and isolation of North African populations. This scenario would explain the greater similarity between lizards from south Spain and North African forms than between southern Iberian and other regions in the Peninsula (Álvarez et al., 2000; Paulo et al., 2002; Batista et al., 2004, Carranza et al., 2004a; Harris et al., 2004; Veith et al., 2004).

1.1.1.1. Southeast Iberian Peninsula: *Podarcis hispanicus*

Lizards that inhabit the SE Iberian Peninsula are the paraphyletic group called *Podarcis hispanicus*. Numerous studies have been carried out with the aim of defining taxonomic units and evolutionary relationships inside this group. Pinho et al. (2006) in a phylogenetic analysis of the *Podarcis* from the Iberian Peninsula and North African described, using a long fragment of mtDNA sequences (2,425 bp), the new form of Galera, represented by a unique sample, from the Baza Depression of SE Spain, as a sister taxon to all other Iberian and North African *Podarcis*. The clustering between this new lineage and *P. liolepis* with high support led to the Galera lineage being considered as a relic of the common ancestor distribution (Pinho et al., 2006). Later work using allozyme markers (Pinho et al., 2007) largely corroborated the subdivisions reported for mtDNA. Reciprocal monophylia of Galera could not be evaluated, because although more individuals from Galera lineage were included, these came from a single location. The genetic differentiation measured based on F_{ST} values between the two forms of *P. hispanicus* (Galera and Valencia lineage) and all the other species showed values comparable with those found between recognized species. One of the phylogenetic relationships well supported in this analysis was the separation between Galera lineage and *P. vaucheri*, with regard to Valencia lineage and *P. liolepis*. Pinho et al. (2007) considered these findings contradictory, because each pair was composed of forms that do not share ancestors according to previous mtDNA phylogeny (Pinho et al., 2006). Nuclear genealogies of two nuclear introns (beta-fibint7 and 6-Pgdint7) failed to define species as monophyletic, and the results obtained suggested that there exists gene flow between forms that are sympatric at least in part of their distribution, such as Galera and Valencia lineages (Geniez et al., 2007; Pinho et al., 2008). Even though gene flow events have been found between *P. vaucheri* and Valencia lineage, these two species still have poorly studied distribution limits, so it is as yet unknown whether they establish contact zones or whether they are completely allopatric. Although gene flow among forms may have occurred, the main cause for species polyphyly is Incomplete Lineage Sorting (ILS), implying that most forms have been isolated since their divergence. Renoult et al. (2009) suggested that three evolutionary lineages could be identified in eastern Iberia using morphological characters and nuclear loci (*P. virescens*, *P. liolepis* and *P. hispanicus* (Galera lineage)), whereas analysis of mtDNA data revealed four lineages (*P. virescens*, *P. hispanicus* (Valencia lineage), *P. liolepis* and *P. hispanicus* (Galera lineage)).

The last and currently accepted phylogeny, which includes SE Iberian forms, is the one elaborated by Kaliontzopoulou et al. (2011). In this study, a second individual belonging to Galera lineage was introduced in the phylogenetic tree and a new lineage was discovered in the SE region of Iberia (Albacete/Murcia lineage). Galera and *P. liolepis* clustered together. Valencia lineage was considered the nominotypical taxon (*P. h. sensu stricto*) and formed a second cluster together with forms from North Africa and SE region of Iberian Peninsula, including the new lineage from Albacete/Murcia. Western and central Iberia forms grouped in the third cluster (Kaliontzopoulou et al., 2011) (Figure 3).

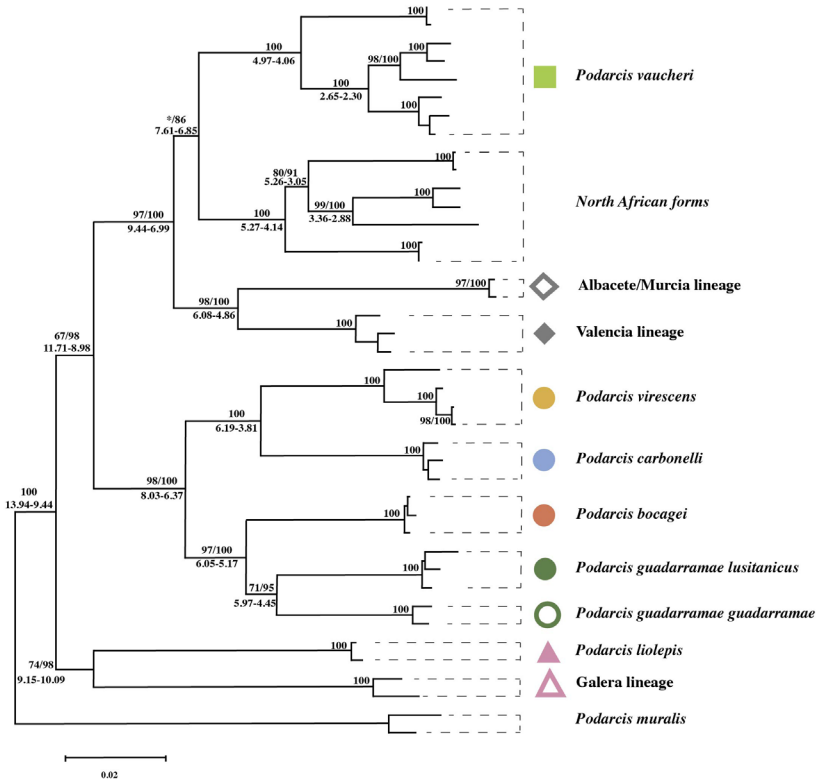


Figure 3. Current phylogeny of *Podarcis* lineages from the Iberian Peninsula and North Africa. Source: Kaliontzopoulou et al. (2011) with modifications.

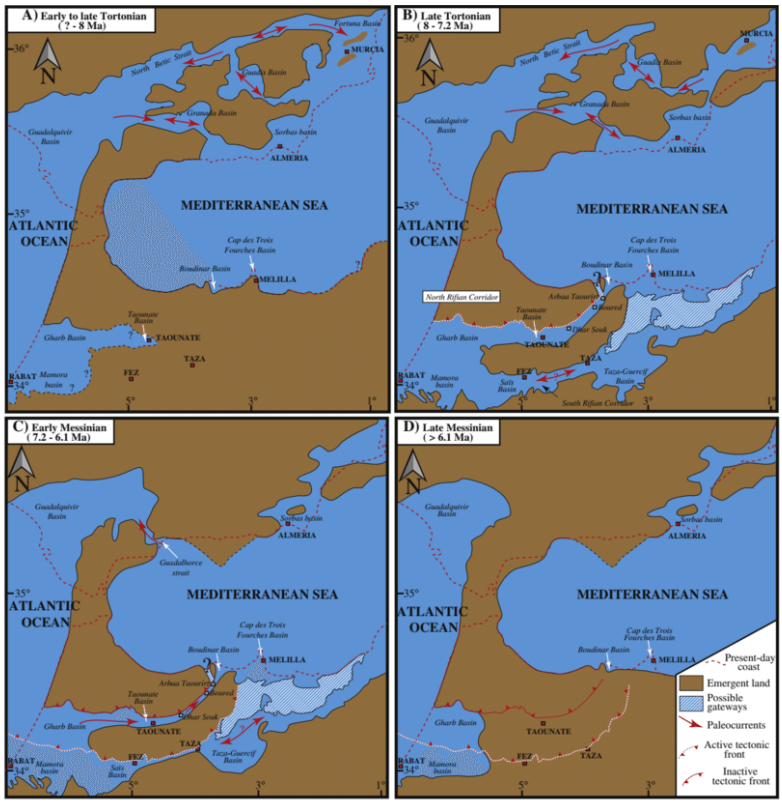


Figure 4. Paleographic maps showing changes in connections between the Iberian Peninsula and North Africa from the early Tortonian to the late Messinian. Source: Achalhi et al. (2016).

1.1.1.2. Columbretes Islands: *Podarcis liolepis*

The Columbretes archipelago (named in the past Columbraira or Ophiusa, terms that allude to the large amount of snakes that populated them until the 19th century, when intensive extermination campaigns started) consists of a group of small islets located to the east of the Iberian Peninsula in the Mediterranean Sea with an approximate area of 19 ha (Castilla et al., 2005).

These uninhabited islets are located between Castellón (Comunitat Valenciana) and Ibiza (Balearic Islands), at 51 and 100 km of distance, respectively (Castilla et al., 1998a) (Figure 5). The geological origin of this archipelago is dated between 1-0.3 Ma, which corresponds to the period after Pleistocene volcanic episodes (Aparicio et al., 1991). These islands have not always been isolated from the mainland, because during the last glaciations, in particular the Würm (mid and low Pleistocene), the Mediterranean Sea level dropped approximately 120 m. The channel between the Iberian Peninsula and the Columbretes appears to have a depth of approximately 90-100 m, hence both territories are likely to have been connected (CLIMAP, 1976).

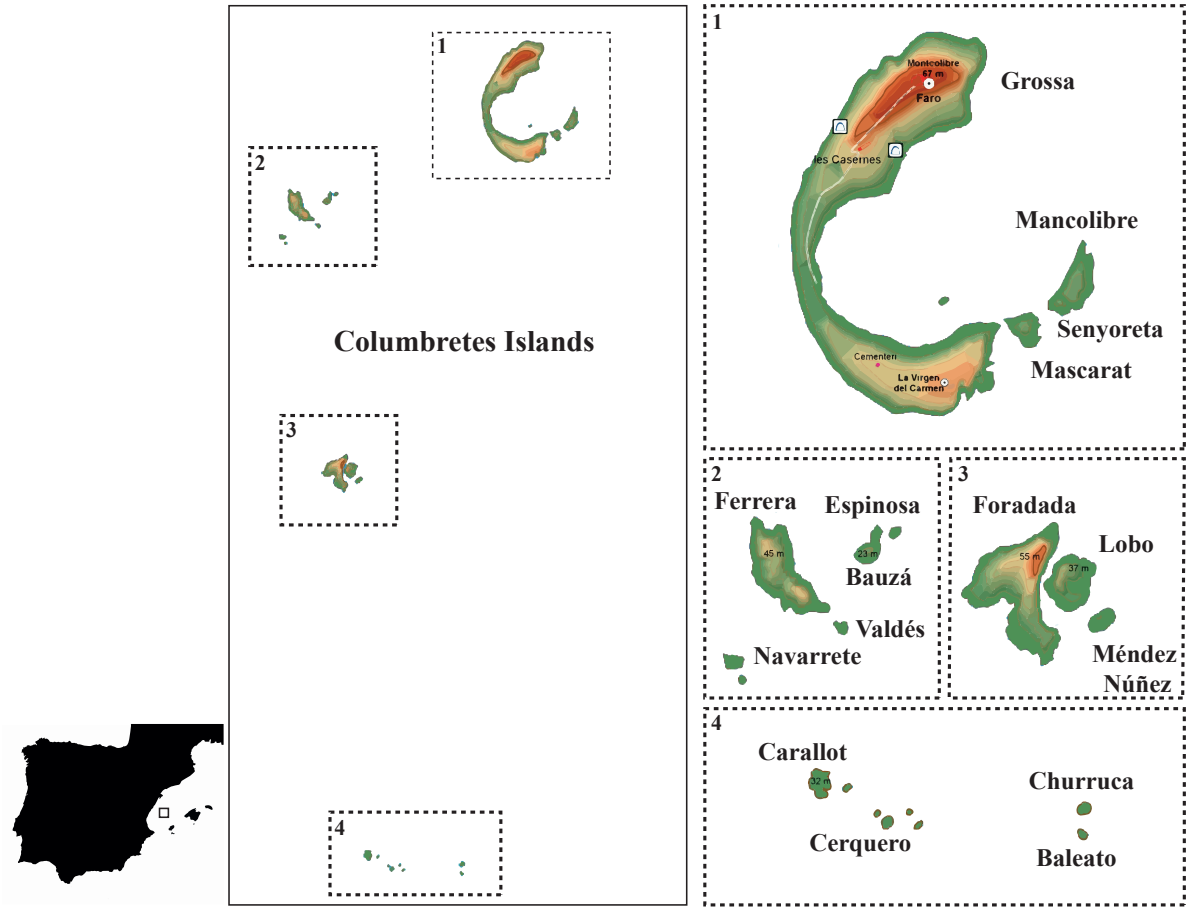


Figure 5. Location of the Columbretes Islands and the principal islets that compose the archipelago.

The archipelago comprises four different groups of islets (Figure 5): 1) Grossa (13 ha), Mascarat, Senyoreta and Mancolibre, 2) Ferrera (1.5 ha), Espinosa, Bauzá, Valdés and Navarrete; 3) Foradada (1.6 ha), Lobo (0.5 ha) and Méndez Núñez; 4) Carallot (0.1 ha), Cerquero, Churruca and Baleato. The archipelago remained uninhabited, except for 1855 to 1975, when lighthouse keepers were present. Some islands (Foradada, Ferrera and Carallot) were used for military exercises that used live ammunition, with effects on the flora and fauna of the islands. Some examples of human impact are the extermination of vipers (*Vipera latasti*) and dramatic reduction of scorpions (*Buthus occitanus*), burning of natural vegetation, and the introduction of domestic species such as pigs, rabbits, cats, and goats. The Columbretes archipelago was declared a nature reserve in 1988 and marine reserve in 1990. Thereby, the conservation and preservation of endemic species became the main goal (Castilla & Bauwens, 1991a, 1991b). The extant terrestrial vertebrate fauna consists of some breeding bird species (*Falco eleonorae, Larus audouinii* or *Hydrobates pelagicus*) and the endemic lizard first named as *Podarcis atrata* and subsequently considered conspecific with *Podarcis liolepis* (Harris et al., 2002, Harris & Sá-Sousa, 2002, Pinho et al., 2006, 2007, 2008, Renoult et al., 2010, Kaliontzopoulou et al., 2011). The Columbretes *Podarcis* ancestor could have arrived from the mainland, during the last glaciation (Würm) about 20,000 years ago. Only in four small islets of the archipelago (Grossa, Mancolibre, Foradada and Lobo) can we find this lizard species, probably because of the limited food resources and the small area of the other islets (Castilla & Bauwens, 1991a, 1991b, 2000). The conservation status of this endemic species is still not well defined, following its recent taxonomic change, although it was considered vulnerable for the national institutions. Habitat fragmentation and the low number of individuals making up these populations are important aspects that influence the survival of this species, which could lead it to extinction (Castilla, 2002; Castilla et al., 2006).

Figure 6. Morphology of *P. liolepis* specimens from the Columbretes Islands (left) and the Iberian Peninsula (Peñagolosa) (right). Source: Valentín Pérez-Mellado.

Individuals from the Columbretes islands were firstly considered part of the *Podarcis hispanicus* complex but were then raised to species rank with the name *Podarcis atrata* (Castilla et al., 1998b). Currently, the *Podarcis* from Columbretes is considered conspecific with *P. liolepis* (Harris & Sá-Sousa, 2002, Renoult et al., 2010, Geniez et al., 2014). Consequently, the status of *P. atrata* as endemism is considered doubtful. The morphologic divergence between *P. liolepis* (Figure 6) from the mainland and from the Columbretes islands is attributable to local adaptation that arose in a very short period (end of the last glacial period) (Castilla & Bauwens, 1997; Harris & Sá-Sousa, 2002; Arntzen & Sá-Sousa, 2007).

1.1.2. Gymnesian Islands' endemic lizard: *Podarcis lilfordi*

The Balearic Islands (Figure 7) are located 90 km from the Iberian Peninsula and form an archipelago of 151 islands and islets. Four of these islands (Mallorca, Menorca, Ibiza, and Formentera) are inhabited and constituted more than 99% of the total surface area (Mannion & Vogiatzakis, 2007; Morey & Ruiz-Pérez, 2008). Mallorca, Menorca, Cabrera, and their surrounding islets are grouped in the Gymnesian Islands, and Ibiza and Formentera and their islets form the Pityusic Islands.

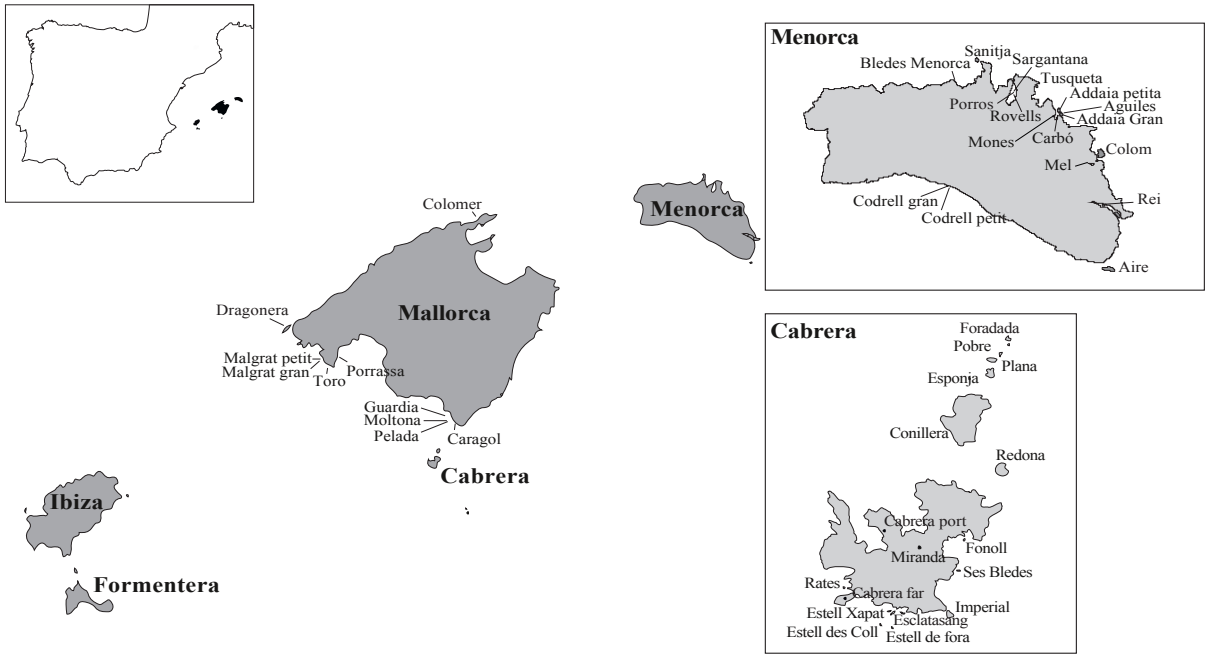


Figure 7. Location of the Balearic archipelago and the primary islands and islets inhabited by endemic lizards.

Podarcis lilfordi (Günter, 1874) inhabits the Gymnesian Islands, although it has disappeared from the largest islands of Mallorca and Menorca (Terrasa et al., 2009a) because of the introduction of predators (marten, genet, weasel, and other domestic carnivores) by humans (Kotsakis, 1981; Corti et al., 1999; Salvador, 2006; Pérez-Mellado, 2009), 2,000 years ago. Twenty-four subspecies have been identified in the 42 known locations, distributed in 10 of the 40 islets of Majorca, and 16 of the 29 in the Menorca and Cabrera archipelago (Pérez-Mellado, 2009; Terrasa et al., 2009a; Pérez-Cembranos et al., 2020). Morphologically, it is a robust lizard, whose head-to-body maximum length is up to 81 mm in males and 75 mm in females, and with different pigmentation patterns (Pérez-Mellado, 1998), with colourations that include brown, green or black. Body size and colour pattern vary depending on the micro-island population to which they belong.

Podarcis lilfordi is classified as an endangered species (EN) according to the IUCN, in the global category (Pérez-Mellado & Martínez-Solano, 2009) and in the national one (Pérez-Mellado, 2002). In the Balearic category, this species is listed as vulnerable. The definition of this category is based on diverse criteria such as population fragmentation, decreasing occupied area, and quality of the habitat. This species is included in the category of special interest in the National Catalogue of Threatened Species, and is also included in Appendix II of the Berna Convention, in relation of the Conservation of Wildlife and Natural Environment in Europe (Salvador, 2006; Viada, 2006).

Regarding the intraspecific diversity of *P. lilfordi*, four mtDNA clades have been validated by phylogenetic and population structure analysis: i) Menorca, ii) western Mallorca, iii) Mallorca (north and south) with north Cabrera, and iv) south Cabrera (Brown et al., 2008; Terrasa et al., 2009a) (Figure 8).

Menorca populations were the first to diverge from the other populations in the Balearic archipelago around 2.6 Ma; and subsequently, approximately 2 Ma, the western Mallorca populations diverged (Brown et al., 2008). Even though different glacial events occurred in this region with drops in sea levels that caused the union of the islands of the Balearic archipelago, no gene flow or migration has been detected between Menorca and Mallorca populations. This lack of introgression could be explained by environmental conditions or selection against hybrids (Terrasa et al., 2009a). Menorca lizard populations show low levels of genetic variability and genetic patterns that suggest a demographic expansion followed by isolation of islet populations due to rises in sea level occurring recently.

Meanwhile, western Mallorca populations present high levels of genetic diversity in comparison with other clades. The clade formed by populations from southern Mallorca and northern Cabrera includes the Colomer population despite their geographical isolation (Figure 9). This clade diverged from the other Cabrera populations around 1.2 Ma. The populations from south Cabrera have an allopatric fragmentation due to the higher sea-depths between southern islets and their divergence dates around ~0.8 Ma (Brown et al., 2008). The sea level fluctuations that occurred during the Quaternary were probably insufficient to reconnect the main islands (Mallorca, Menorca and Cabrera) but were able to allow repeated connections between islet populations separated by shallow channels (Terrasa et al., 2009a) (Figure 10).

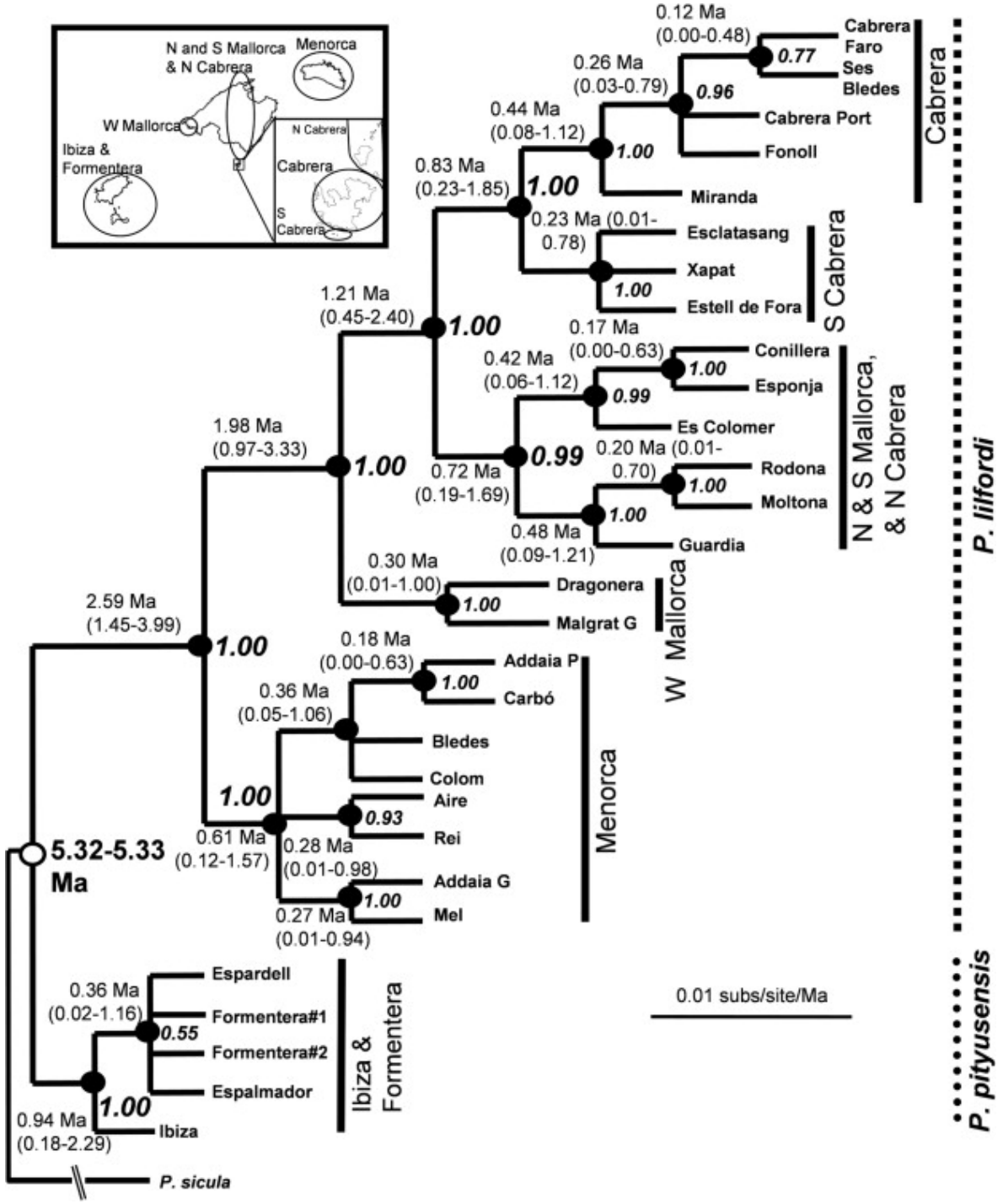


Figure 8. Bayesian phylogeny of *P. lilfordi* indicating the principal clades and divergence times. Source: Brown et al. (2008). Abbreviations: S, south; N, north; W, west.

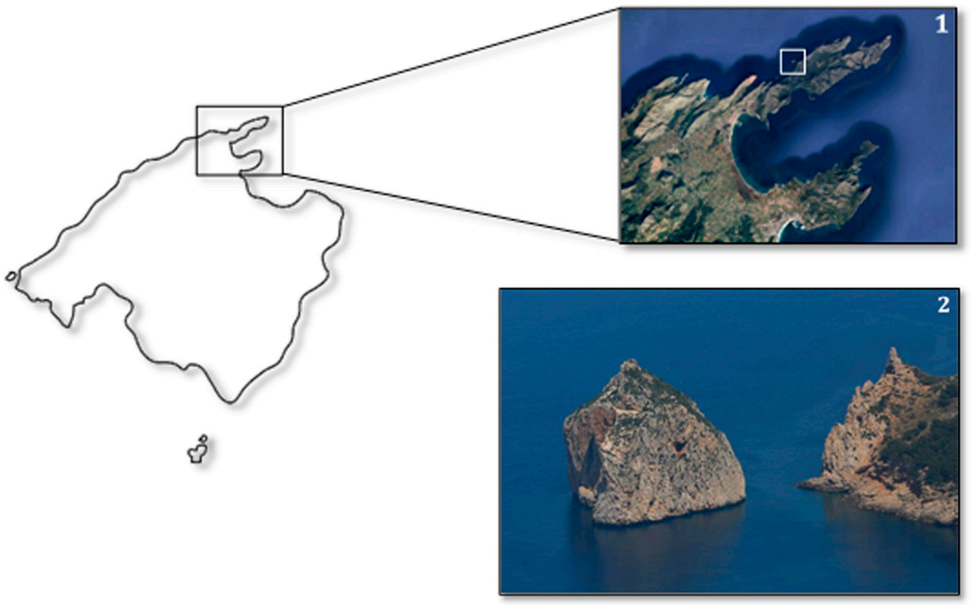


Figure 9. Location of Colomer Island, in Mallorca (1) and its characteristic orography and geographical isolation (2).

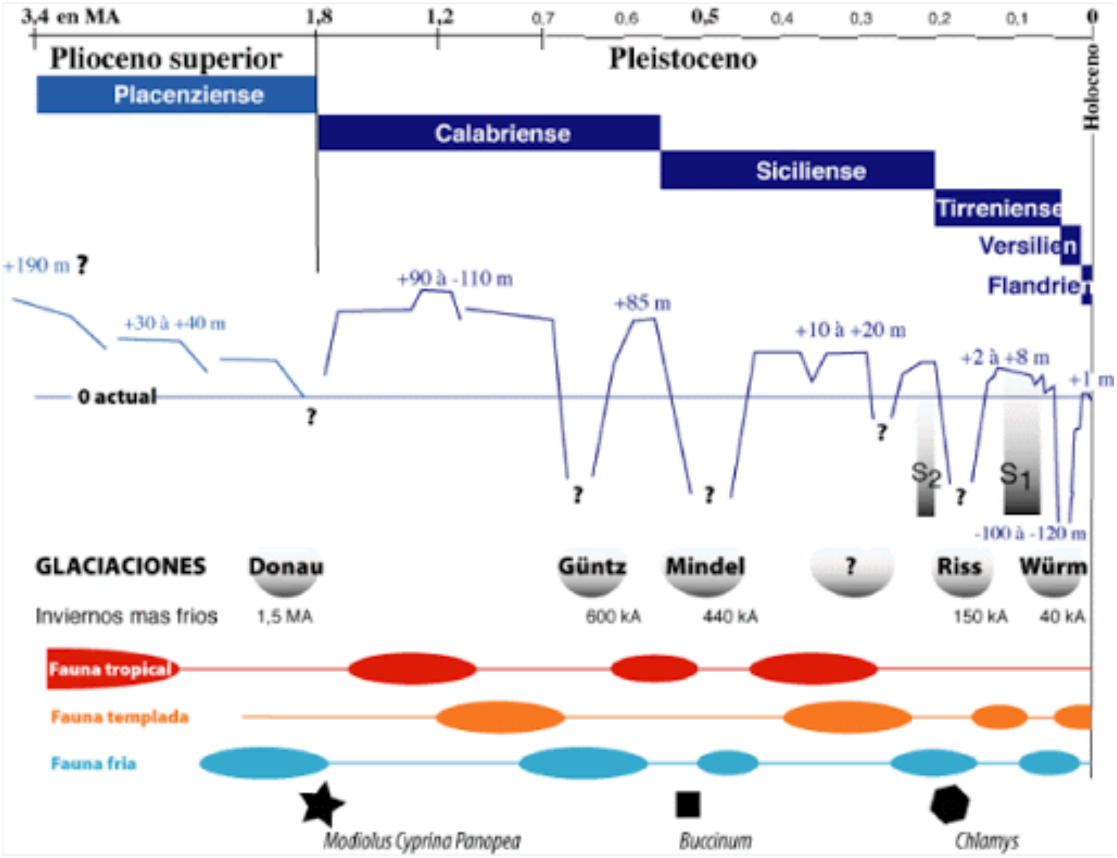


Figure 10. Sea level fluctuations during the Quaternary glaciations in the Mediterranean Sea. Source: Emig & Geistdoerfer (2004).

1.2. Evolutionary mechanisms of divergence

Evolution is the process of biological change over time in natural populations. Such variability, especially at the genetic level, is influenced by different mechanisms that increase or decrease genetic variation, including natural selection, genetic drift, gene flow, and mutations (Gavrilets, 2014). Evaluation of this genetic variability and the determination of population structure are important tools to identify different species and the phylogenetic relationships between and among them, in order to consequently develop effective conservation approaches (Coates et al., 2018).

1.2.1. Divergent selection

Divergent selection consists of the accumulation of differences between closely related species, resulting in the formation of new species (speciation). Divergent selection occurs when two groups within a population experience isolation and go on to undergo different selective pressures in order to respond to changes in abiotic factors, such as environmental conditions, or biotic factors (i.e. presence or absence of predators). The frequency of genes related to survival and reproduction increase or decrease since gene flow is restricted.

Several generations later, continual evolution brings about reproductive isolation between the populations (Gulick, 1888; Bergstrom & Dugatkin, 2016). There are several types of speciation depending on the type of reproductive barrier, which produces the formation of different species within a population (Figure 11):

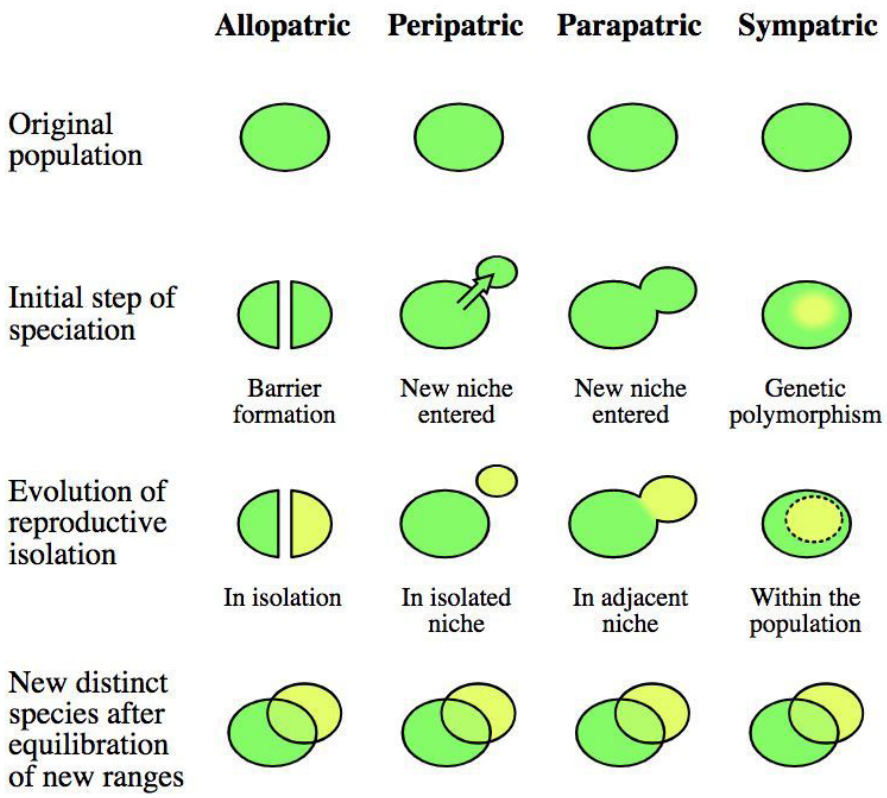


Figure 11. The 4 basic models of speciation: allopatric, peripatric, parapatric, and sympatric. Source: https://www.nationalgeographic.org/encyclopedia/speciation/

Allopatric speciation occurs when a physical or geographical barrier separates species into two groups that become reproductively incompatible due to their isolation. The development of new species depends on the characteristic habitat or genetic traits of the group that are inherited. In peripatric speciation, small groups of individuals separate from the larger group and form a new species. As in allopatric speciation, physical barriers cause the reproductive isolation and genetic differentiation of the small group. In parapatric speciation, speciation occurs between adjacent populations occupying an extensively continuous habitat. Speciation occurs due the different characteristics in the same environment. A single mutation or localised adaptation are mechanisms that can produce divergence among parapatric populations. Sympatric speciation occurs when proximal individuals with no physical barriers specialise in different resources. This type of speciation could be explained by competition for ecological resources, competition for mates, and habitat-specific deleterious or beneficial alleles. However, the most common scenario involves disruptive selection, that is, natural selection acting in contrasting directions within a population, each with different resource specialization, favouring extreme phenotypes and reduced hybrid fitness (Rundle & Nosil, 2005; Coyne, 2007).

1.2.1.1. Sources of divergent selection

Environmental differences

A primary source of divergent selection is the different environmental characteristics between populations, such as habitat structure, climate, resources, and the presence or absence of predators or competitors (Schluter, 2000). Environmental factors have been involved in the development of reproductive isolation in some cases of ecological speciation (Nagel & Schluter, 1998; Via et al., 2000; Jiggins et al., 2001; Linn et al., 2003), but there are other factors that have been less studied. For example, predation is present in natural populations and adaptation to it may have important consequences for reproductive isolation. However, divergent selection related to predation or competition has been the object of study in few cases (Jiggins et al., 2001; Vamosi & Schluter, 2002; Nosil, 2004).

Sexual selection

The second ecological source of divergent selection involves sexual selection, which may be a powerful force in the evolution of reproductive isolation, because it acts on traits involved in mate recognition (Panhuis et al., 2001). Mating preferences can evolve because of divergent selection between environments or not (Schluter, 2000, 2001; Boughman, 2002). Models involving divergent selection include spatial variation in natural selection on secondary sexual traits (Lande, 1982) and on mating or communication systems (Ryan & Rand, 1993; Boughman, 2002). Examples that do not involve divergent selection between environments are models in which sexual selection emerges from the interaction of sexes (i.e. Fisher's runaway) (Lande, 1981) and sexual conflict (Chapman et al., 2003).

Ecological interactions

Divergent selection may also arise because of ecological interaction between populations, depending on the frequencies of different phenotypes (Taper & Case, 1992; Schluter, 2000). Interspecific competition is the most common ecological interaction in nature, although there is no research linking reproductive isolation with interspecific competition. Other types of ecological interactions, such as mutualism, facilitation, or apparent competition (Abrams, 2000; Doebeli & Dieckmann, 2000; Day & Young, 2004), may also cause divergent selection, but its role in ecological speciation is unexplored.

1.2.2. Genetic drift

Genetic drift is a stochastic process that occurs by chance in nature and influences or changes allele frequency within a population because of sampling error from generation to generation. Some alleles can be completely lost within a generation due to genetic drift, even if they are beneficial traits to evolutionary success.

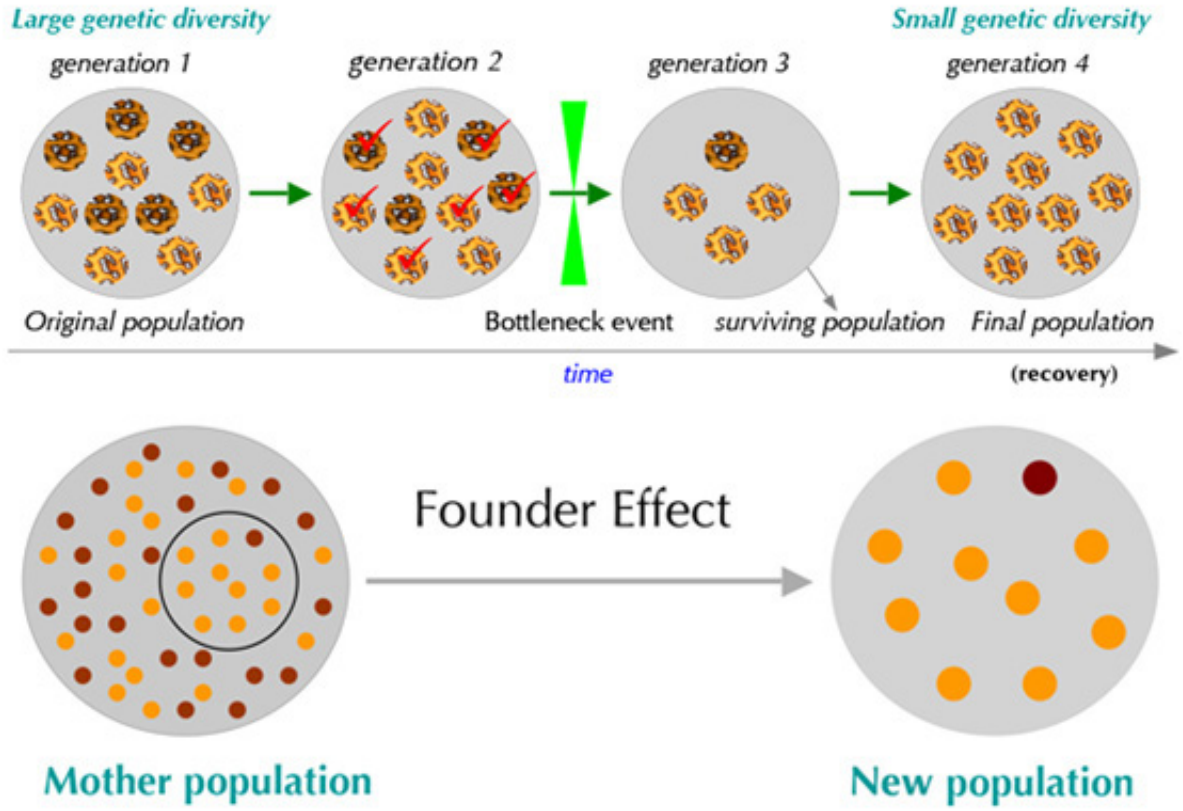


Figure 12. Graphics of population events that speed up genetic drift: bottleneck and founder effect. Source: Cosnet Lab https://nectunt.bifi.es/to-learn-more-overview/mechanisms-of-evolutionary-change/

The unique effect of genetic drift can be enough to drive genetic variability due to variation in allele frequencies between populations, consequently leading to population subdivision (Lande, 1976; Coyne & Orr, 2004). The effect of genetic drift is more notable in small populations, because deleterious mutations are expected to drift to fixation (Kimura & Crow, 1964; Kimura, 1968). Some population events, such as bottlenecks (Mayr, 1966; Carson, 1971) or founder effect can speed up the effect of genetic drift (Huxley et al., 1958; Barton & Charlesworth, 1984). A bottleneck occurs when there is a strong decline in a population's size, commonly due to environmental factors. It is an event in which some genes are extinguished from the population, provoking a drastic reduction of genetic diversity of the original gene pool. Founder effect occurs when a new population is founded by a small number of individuals, entailing a loss of genetic diversity (Figure 12).

1.2.3. Evolution in insular populations

Islands are global centres of biodiversity and endemism (Stuart et al., 2012), and are therefore very important for the field of Evolutionary and Conservation Biology (Kaneshiro, 1995). Insular populations are naturally isolated systems and have become unique experimental models for evolutionary biologists (Grant, 1998; Whittaker & Fernández-Palacios, 2007; Losos & Ricklefs, 2009). Isolation is a feature commonly associated with divergence and speciation among island populations (Grant, 1998), but the evolutionary processes of genetic drift and/or divergent selection acting on genetic variability and new mutations are necessary for the accumulation of differences (Fisher, 1930; Wright, 1931, 1951). There is more research on island biodiversity focused on divergent selection causing adaptive evolution (Losos et al., 1998; Grant & Grant, 2002), than studies focused on the role of genetic drift in generating differences between isolated populations (Jordan & Snell, 2008). Thus, investigating the joint roles of genetic drift and divergent selection is key to understanding how island populations diverge, thereby generating island biodiversity and endemism.

Genetic drift is expected to be strong in insular populations for several reasons. First, many island populations are founded by a small number of individuals (founder effect) whose genetic composition may differ from the original population due to random chance (Martínez-Solano & Lawson, 2009; Kolbe et al., 2012). Second, insular populations usually have small effective population sizes (Ne), related to island size and carrying capacity (Frankham, 1998; Eldridge et al., 1999). The third reason is bottlenecks, i.e. fluctuations in population size in the course of their history, resulting in population size reductions (Frankham, 1998; Heber et al., 2013). Isolated island populations may not receive an infusion of genetic variation through gene flow after bottlenecks, contrary to continental populations, deriving in a permanent reduction in genetic variation (or a long-term reduction, as mutation may eventually replenish lost genetic variation) (Eldridge et al., 1999).

Divergent selection is expected to be powerful among island populations due to their characteristic isolation and the high environmental diversity between islands and between islands and the mainland (Weigelt et al., 2013). Different environmental factors are involved in this habitat variability: climate variables, such as temperature or precipitation, or differences in elevation and topography (Fischer & Still, 2007; Spalding et al., 2007).

The island biogeography theory makes the important prediction that larger islands and islands closer to the mainland have a lower extinction rate and higher immigration rate than smaller, isolated ones, resulting in greater species diversity (MacArthur & Wilson, 1967; Power, 1972; Simpson, 1974; Frankham, 1997; Kalmar & Currie, 2006; McGlaughlin et al., 2014; Wang et al., 2014; Băncilă & Arntzen, 2016). However, even adjacent islands that are the same size may have different species due to climate, microhabitat availability, and the random chance of which species end up on which islands (Burns, 2007). The combined role of genetic drift and divergent selection could drive genetic differentiation and population divergence among island populations, although strong drift may swamp selection, preventing adaptive divergence (Wright, 1931, 1951).

1.2.4. Tools for analysis of genetic divergence: Molecular markers

The huge diversity exhibited by the different organisms that inhabit the world can be reflected not only in phenotypic characters but also at the molecular level. Phylogenetic relationships, genetic structure, and evolutionary history among organisms or genes are studied by comparing DNA or protein sequences. Differences between the sequences evidence genetic divergence caused by evolutionary processes over time.

Despite the large number of species described, there are still millions of unclassified or unknown species. The traditional system of organism classification is based on morphology and presents some limitations. Molecular markers are also useful in the classification and identification of unknown organisms. Their use, despite presenting some weaknesses, can complement the traditional morphology-based method for ecological studies (Patwardhan et al., 2014).

Next-generation sequencing (NGS) technologies are revolutionising the field of evolutionary biology, providing new opportunities for genetic analysis at scales not previously possible. These new technologies open the possibility to carry out research in population genetics (Hohenlohe et al., 2010), quantitative trait mapping (Baird et al., 2008), comparative genomics, and phylogeography (Emerson et al., 2010; Gompert et al., 2010) at a genome-wide level in model and non-model organisms (Mardis, 2008a, 2008b). Despite considerable progress, these techniques have some limitations, mainly related to the need to develop robust analytical tools to carry out the bioinformatic analysis (Etter et al., 2012).

In general, molecular markers play a basic role in the establishment of genetic variation and biodiversity with precision and reliability. These markers can be mainly classified into two types: mitochondrial and nuclear markers.

1.2.4.1. Mitochondrial DNA

Until now, mtDNA has been the most used molecular marker; therefore, there is an advanced development in techniques and methodology (Patwardhan et al., 2014). It encompasses several features that make it optimal, such as its molecular simplicity, high levels of variability and almost neutral mode of evolution (Avise, 2004, 2009). Mitochondrial markers also have an effective population size (Ne) approximately one-quarter that of nuclear markers, making it possible to recover the pattern and time of recent historical events; since due to the low recombination present in this genome region, the whole molecule can be assumed to have the same genealogical history (Castro et al., 1998; Jiang et al., 2016). The variable substitution rates enable faster evolving regions of the mitochondrial genome to be used for intraspecific variation, and the slower evolving regions for interspecific or intra-genera variation (Gübitz et al., 2000, 2005; Brown et al., 2000, 2006, 2008; Amato et al., 2008; Terrasa et al., 2009a).

Even though mitochondrial DNA has been proved to be extremely useful in describing population genetic structure, resolving species-level phylogenies, or phylogeographic analysis, it has limitations. It only provides information from the maternal lineage and does not recombine, meaning that the resulting gene tree might have a different history to the information that could be obtained with a genomic approach. Besides, there have been technical issues arising from the presence mtDNA integrated into the nuclear genome that could lead to analysis error (Hurst & Jiggins, 2005).

The vertebrate mitochondrial genome is a circular molecule about 17 kb in length containing 37 genes (Wolstenholme, 1992). Among these, there are two ribosomal RNA (rRNA) genes: 12S and 16S. 12S rRNA is highly conserved and has been applied to understand the genetic diversity of higher categorical levels such as phyla. Meanwhile, 16S rRNA is often used for studies at family or genus levels (Gerber et al., 2001).

Mitochondrial coding genes are regarded as powerful markers for genetic diversity analysis at lower categorical levels, due to their faster evolutionary rates compared to rRNA genes. Animal mitochondria contain 13 protein-coding genes; however, three of the most extensively used are cytochrome b (CYTB), NADH dehydrogenase (NADH), and mitochondrial cytochrome oxidase I (COI). COI has recently gained more attention in developing DNA barcodes for species identification and biodiversity analysis (Janzen et al., 2005; Dawnay et al., 2007). Mitochondrial DNA also contains a non–coding region called the control region (CR) due to its role in replication and transcription of mtDNA. The CR fragment shows a higher level of variation than coding sequences due to reduced functional constraints and relaxed selection pressure (Onuma et al., 2006; Arif & Khan, 2009).

1.2.4.2. Nuclear DNA

The second type of markers, as essential as mitochondrial DNA, are nuclear loci, which represent bi-paternal inheritance (Jiang et al., 2016). The most used nuclear markers include random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites, and single nucleotide polymorphisms (SNPs). These markers have several applications in biodiversity analysis and are very useful in determining genetic variability among individuals by comparing genotypes at a number of polymorphic loci (Avise, 2004).

Random amplified polymorphic DNA (RAPD) markers use PCR to amplify random segments of nuclear DNA. The technique uses short single primers that attach to both strands of DNA and low annealing temperatures with the aim of amplifying multiple regions (multilocus). The major limitation of RAPD is the inability to differentiate between homozygote and heterozygote, although it is a simple and inexpensive technique.

Amplified fragment length polymorphism (AFLP) is a multilocus technique that includes restriction digestion and PCR amplification. The main advantages of AFLP are its high specificity and reproducibility since it allows for selective amplification due to the use of restriction digestion, specific adaptors, and high annealing temperatures.

Microsatellites are multiple copies of short tandem repeats, located in both coding and non–coding regions and distributed throughout the genomes. Microsatellites are markers with multiallelic presentation in different populations.

The development of more advanced sequencing techniques has enabled the emergence of SNPs. These markers enable single base differences to be detected between several sequences of an individual region or of a whole genome within species. Depending on the level of DNA sequencing (genome-wide or specific region), SNPs can provide broad genome coverage, high levels of variability, and can be used for phylogenetic reconstruction (Arif & Khan, 2009).

1.2.4.2.1. Single Nucleotide Polymorphisms

Single nucleotide polymorphisms are single base changes in the nucleotide sequence of genomic DNA that may result in a phenotypic characteristic or not. SNPs are usually biallelic markers that present less variability than microsatellites, but they are important nuclear markers that have been widely used for population structure and genetic diversity studies due to the large increase in the number of loci available (Brumfield et al., 2003) and the high polymorphism among populations or individuals (Morin et al., 2004). Other advantages are their simpler mutational process that causes a lowered rate of homoplasy, and the capacity for rapid, large scale, cost-effective genotyping (Syvänen, 2001; Vignal et al., 2002; Brumfield et al., 2003; Chen & Sullivan, 2003; Schlötterer, 2004). Several studies have examined the theoretical potential of SNPs for estimating parameters such as population history and inference of pairwise relationships (Kuhner et al., 2000; Glaubitz et al., 2003), and their application in ecological or conservation analysis (Morin et al., 2004; Kleinman-Ruiz et al., 2017; Rhode et al., 2017; Lemopoulos et al., 2019).

1.2.4.2.2. Genome-wide sequencing: ddRAD sequencing

The relatively newly developed method that generates short sequenced segments throughout the whole genome, to be subsequently analysed and compiled into genomes, is called Next Generation Sequencing (NGS). NGS technologies have become an important tool for molecular ecologists interested in performing evolutionary, ecological, and conservation studies (Allan & Max, 2010), since they open the possibility to carry out genetic analysis at a genomic scale and with non-model organisms, meaning individuals with few genomic resources (Etter et al., 2012). Studies based on large, genome-wide datasets improve the power of studies based on a small number of neutral loci in the determination of population structure; as well as in the estimation of the small proportion of loci that are putatively to be under selection and consequently ecologically relevant in adaptation (Allendorf et al., 2010; Stapley et al., 2010; Narum et al., 2013). With these new genome-wide techniques, we can assess levels of population structure not detected with previous sequencing techniques. Recent episodes of divergence, high gene flow, or detection genetic drift can be observed (Benestan et al., 2015; Lal et al., 2016; Vendrami et al., 2017), and, even when there is negligible neutral differentiation (Jones et al., 2012; Pavey et al., 2015), also the detection of adaptive divergence, which is relevant in management decisions and delimitation of conservation units (Funk et al., 2012).

Restriction site associated DNA (RADseq) genotyping methods (Baird et al., 2008; Etter et al., 2012) combined with NGS technologies have become a powerful and widely used tool in ecological and evolutionary genomic studies (Davey & Blaxter, 2010; Andrews et al., 2016). It enables thousands of genome-wide polymorphic sites (SNPs) to be recovered cost-effectively in model and non-model organisms (Davey et al., 2011). The RADseq approach combines a restriction enzyme (RE) digestion and genome-wide sequencing of the regions adjacent to restriction sites, enabling the exploration of homologous genomic regions for thousands of individuals and the identification of several genetic polymorphisms along the genome. Although a larger proportion of the genome could be examined with other NGS methods, they are more expensive and cannot be used with so many individuals (Andrews et al., 2016).

A recent RADseq based genotyping method is double digest RADseq (ddRAD) (Peterson et al., 2012). This technique involves the digestion of genomic DNA using both common and rare REs. After whole genome digestion, different molecular processes such as adaptor ligation and size selection transform DNA fragments into a genomic library suitable for sequencing on a NGS platform. Single-end or paired-end sequencing can be used to generate the amount of genomic information and markers (Figure 13). This protocol differs from RADseq in two principal points: digestion with two REs, rather than random shearing, and the precise size-selection step. These characteristics enable the production of sequencing libraries consisting only of the subset of genomic restriction digest fragments generated by cuts with both REs and which fall within the size-selection window (Peterson et al., 2012).

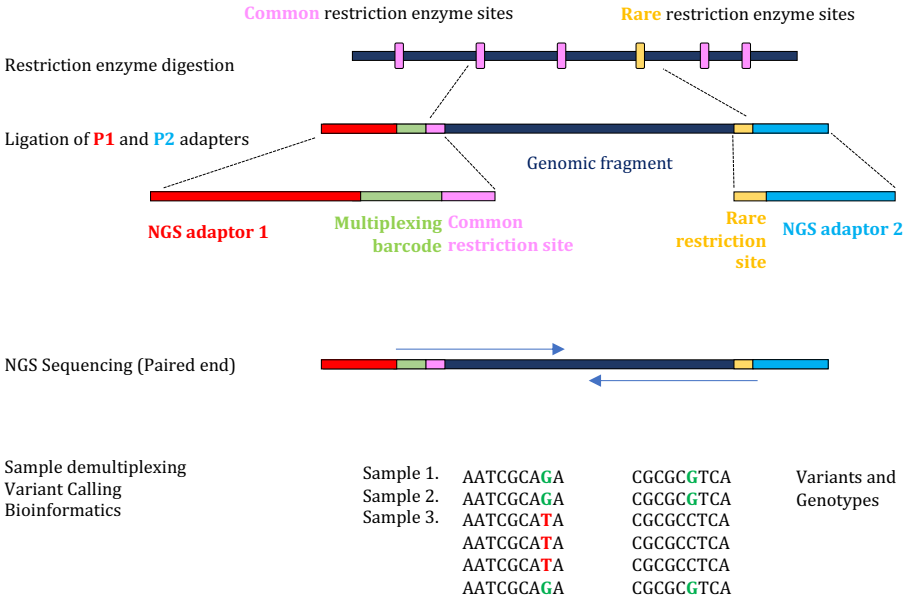


Figure 13. Graphic representation of the ddRAD sequencing process.

1.2.4.2.3. Outlier loci

Determining the genetic basis of adaptive characteristics in natural populations is key to understanding populations' adaptation to variable environments (Nunes et al., 2011a). Diversity in environmental factors may result in phenotypical or physiological differences, leading to morphological and molecular adaptations. Genetic divergence between populations can derive in allele frequency differences at loci related to local adaptation or natural selection, and consequently some regions undergo faster divergence than other regions (Wu, 2001; Nosil et al., 2009). These differences result in the existence of peaks of divergence where genetic differentiation accumulates, and other regions with little to no differentiation (Feder et al., 2013; Seehausen et al., 2014). The regions that exhibit greater differentiation than expected under neutrality are known as "genomic islands" (Figure 14), and are composed of selected, tightly linked loci identified as outliers (Nosil et al., 2009).

Genome-wide approaches make it possible to detect these "genomic islands", which generally have important functions (Nielsen, 2005). Different evolutionary forces such as genetic drift or gene flow, influence all genomic regions, whereas selection leaves a characteristic variability pattern on select loci, enabling the identification of these loci (Beaumont, 2005; Storz, 2005). Detection of these outlier loci is carried out by the estimation of population genetic differentiation (i.e., F_{ST}) (Beaumont & Balding, 2004; Storz, 2005; Bonin, 2008; Foll & Gaggiotti, 2008; Narum & Hess, 2011). Divergence of recently isolated populations may not be reflected at neutral loci and may not be detected by traditional approaches.

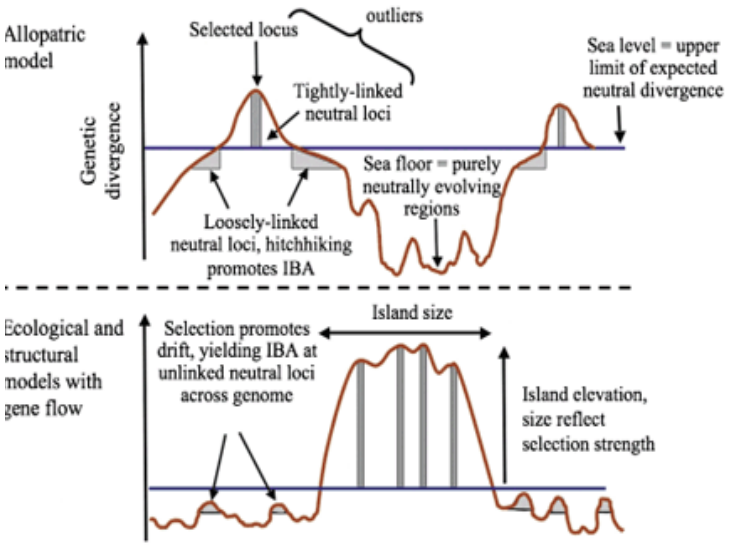


Figure 14. Genomic islands of speciation. Schematic figure of patterns of differentiation along a chromosome. Excavations represent regions under balancing selection, sea floor represents neutrally evolving regions, and sea level represents a neutrality threshold. Islands are regions with greater differentiation than expected under neutrality. Source: Nosil et al. (2009).

Otherwise, loci putatively under selection may offer valuable population markers for more recent ecological timescales (Russello et al., 2012). Genetic variation at neutral loci is shaped by mutation, recombination, gene flow, and genetic drift (Wright, 1931), and has effects on genome-wide variation within and between populations. Natural selection operates on population structure to cause adaptive divergence. Next-generation sequencing is actually the chosen method in population genetics, as it integrates information from neutral and adaptive loci to characterize population genetic structure and adaptive differentiation within populations (Funk et al., 2012).

1.3. Melanism

Melanism is a type of colour polymorphism that consists of completely black coloured individuals; it is a well-studied trait in different taxa and has been the focus of several studies on evolutionary adaptation (Norris & Lowe, 1964; Wiens, 1999; Cox & John-Alder, 2005; Janse van Rensburg et al., 2009; Alho et al., 2010). The role of melanism is very complex and has been related to a wide range of adaptive functions such as sexual selection and reproductive success (Wiernasz, 1989; Sinervo & Lively, 1996; Jawor & Breitwisch, 2003; Griffith et al., 2006; Ducrest et al., 2008; Fedorka et al., 2013).

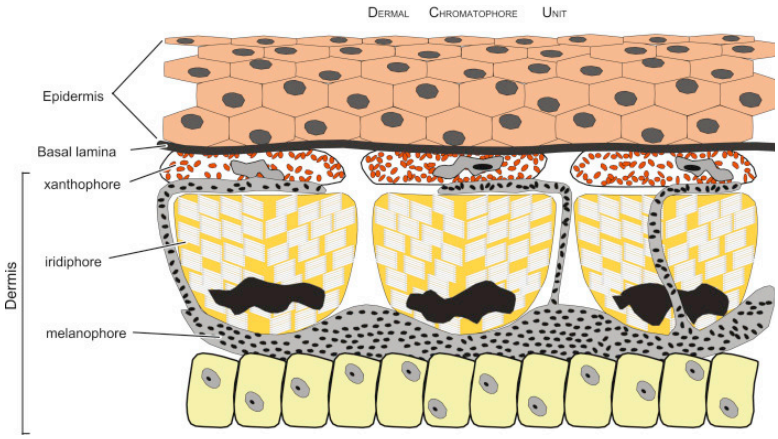


Figure 15. Dermal chromatophore unit. Source: Vitt & Caldwell (2014).

Different physiological functions have also been associated with melanism: thermoregulation, whereby darker animals warm faster and maintain higher body temperature (Kettlewell, 1973; Kingsolver & Wiernasz, 1991; Vences et al., 2002; Clusella-Trullas et al., 2007; Reguera et al., 2014; Azócar et al., 2016); ultraviolet (UV) protection, by discarding harmful radiation with dark pigments (Gunn, 1998; Hofer & Mokri, 2000; Callaghan et al., 2004; Calbó et al., 2005; Reguera et al., 2014); and immune response, taking advantage of the properties of melanin (Mackintosh, 2001; Wilson et al., 2001; Dubovskiy et al., 2013). Other functions like stress resistance, energy balance (Hoekstra, 2006; Ducrest et al., 2008), and crypsis in response to predation risk (Kettlewell, 1973; Endler, 1984; Vroonen et al., 2012; Fulgione et al., 2014; Reguera et al., 2014) have also been related.

In colour-changing vertebrates (except birds and mammals), there are three types of chromatophores (pigment cells): melanophores which impart brown, black, or red colouration; xanthophores, which contain yellow, red, and orange pigments; and iridophores that comprise blue, purple, green, and iridescent pigments (Figure 15) (Hofreiter & Schöneberg, 2010; Kuriyama et al., 2013; Vitt & Caldwell, 2014).

The melanophore's distribution depends on the organisation of melanosomes, which are organelles that exclusively contain melanin (Bagnara & Hadley, 1973), the name given to all members of the tyrosine-derived class of pigments found in melanophores (Lerner & Fitzpatrick, 1950; Nicolaus & Piattelli, 1962; Ito & Wakamatsu, 2003). Melanin can be classified in several types: neuromelanin, allomelanin, pheomelanin, and eumelanin (Fedorow et al., 2005). Neuromelanin is specifically expressed in the nervous systems of primates (Marsden, 1961; Fedorow et al., 2005), while allomelanin is found in fungi, plant, and bacteria kingdoms (Fedorow et al., 2005). Pheomelanin ranges from yellow to red and has been found only in melanocytes of mammalian and avian species (Ito & Wakamatsu, 2003). Finally, there is the brown-black eumelanin, which is produced by all vertebrates with the physiological ability of colour change (Aspengren et al., 2009). In general, variation in colouration in vertebrates is mostly attributable to differential accumulation of reddish-brown pheomelanin and to black-grey eumelanin pigments (Majerus, 1998) (Figure 16).

In reptiles, all three dermal pigment cell types are present in melanic individuals, but melanophores are more abundant (Kuriyama et al., 2016). Reptile melanophores are known only to produce eumelanin (Bagnara & Hadley, 1973), but pheomelanin has also recently been discovered in the shell of Hermann's tortoise (Roulin et al., 2013). Changes in the production and dispersion of melanin granules are ultimately responsible for changes in the dorsal colour of reptiles (Hadley, 1997).

1.3.1. Candidate genes: MC1R

The process of producing melanin (melanogenesis) includes several routes with different genes involved, a fact that makes the task of identifying the base of colouration very complex. The melanocortin 1 receptor (MC1R) is a key protein in this process, controlling the synthesis of melanin through melanocytes (Robbins et al., 1993; Takeuchi et al., 1996). MC1R functions are controlled by the agouti-melanocortin 1 receptor pathway (Figure 16) that modulates the amount and type of pigment produced by melanocytes. MC1R functions are controlled by agonists (alpha-Melanocyte-stimulating hormone (alfaMSH) and Adrenocorticotropic hormone (ACTH)) and antagonists (Agouti-signaling protein (ASIP)). When MC1R is activated by agonists, the production of melanin is stimulated (Yamaguchi et al., 2007).

AlfaMSH activates MC1R provoking an increase in cyclic adenosine monophosphate (cAMP) and eumelanin levels (Nachman et al., 2003). On the other hand, the reduction of AMPc activity provokes greater synthesis of pheomelanin (Kingsley et al., 2009). The presence of ASIP antagonist produces the inhibition of MC1R, causing AMPc levels to decrease, and melanocytes to stop the production of eumelanin and produce pheomelanin (Hoekstra, 2006). The absence of ASIP allows basal levels of MC1R and the activation of eumelanin production. As reptiles are thought to produce only eumelanin but not pheomelanin, MC1R activity might simply affect the amount of melanin produced (rather than switching between the two types of melanin) (Rosenblum et al., 2004).

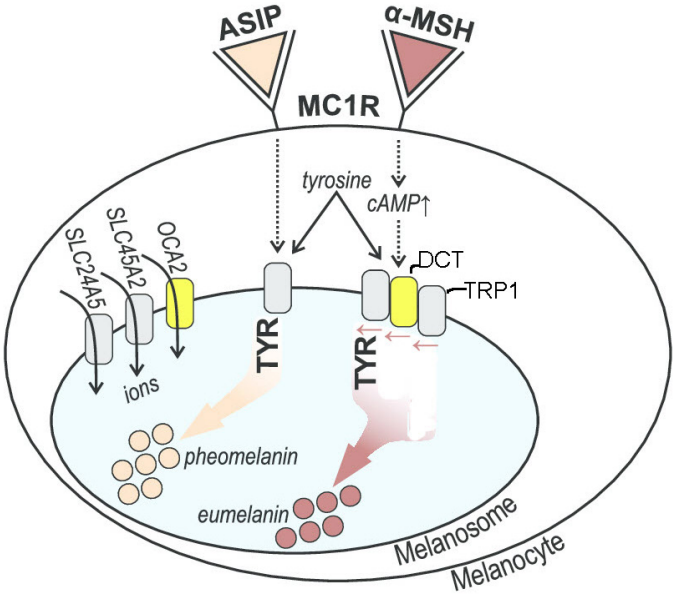


Figure 16. MC1R-ASIP signalling pathway. Source: http://www.evo-ed.org/Pages/Skin/genesproteins.html. Abbreviations: ASIP, agouti-signaling protein; MC1R, melanocortin 1 receptor; alpha-MSH, alpha-melanocyte-stimulating hormone; cAMP, cyclic adenosine monophosphate; DCT, dopachrome tautomerase; TRP1, tyrosinase-related protein 1; TYR, tyrosinase; OCA2, oculocutaneous albinism type II; SLC45A2/SLC24A5, solute carrier family 45 member 2/ solute carrier family 24 member 5.

The structure of these genes has been conserved over a very long period (approximately 400 million years) (Schioth et al., 2005). Gain of function and/or deletion mutations in the MC1R locus have been associated with the presence of melanism (Anderson et al., 2009). In squirrels, for instance, melanism was explained by a deletion in the MC1R locus (McRobie et al., 2009); in birds, an abundant polymorphism in MC1R was reported and associated with black plumage (Guo et al., 2010); and different mutations in the MC1R gene were responsible for the brown colouration of cavefish (Gross et al., 2009).

Several studies have associated MC1R gene and colour polymorphism in amphibians and reptiles (Rosenblum et al., 2010; Nunes et al., 2011b; Fulgione et al., 2015), whereas other studies have found no relation between MC1R and melanism (Herczeg et al., 2010; Corso et al., 2012; Micheletti et al., 2012; Buades et al., 2013).

In the genus *Podarcis*, several melanic populations have been found within different species. This trait was originally thought to be associated with older island populations (Eisentraut, 1949, 1954; Kramer, 1949). In his study, Eisentraut (1954) described melanic populations of *P. lilfordi* from the Foradada island in the Cabrera archipelago, and from the island of Aire, located in Menorca, and, also of *P. pityusensis* from Bleda Plana (Ibiza) (Eisentraut, 1949), hypothesising that dark phenotypes were related to a higher intake of plant material. Kramer (1949), on the other hand, suggested an evolutionary explanation, associating melanism with adaptive advantages through protection from harmful UV radiation, while enhancing heat absorption during cooler weather. Hartmann (1953) proposed that mutations related to melanism were present in the coastal islet populations before their separation from the main islands. Pérez-Mellado (1998) well established the high level of phenotypic variation (body size and colouration) among lizard populations, explaining it as an adaptation to the specific climate of coastal islets.

According to Buades et al. (2013), *P. lilfordi* populations present higher levels of diversity in MC1R gene than in *P. pityusensis*. They also seem to reflect the patterns previously observed in mtDNA and the historical biogeography of these species (Brown et al., 2008; Terrasa et al., 2009a), but no relationship was found between MC1R polymorphism and differences in colouration in Balearic *Podarcis*.

1.3.2. Gene expression

In addition to mutation studies in genes associated with melanism, studies of gene expression, especially with MC1R gene, have also been carried out with differing results. In macaques (Bradley et al., 2013), sheep (Peñagaricano et al., 2012), raccoon dogs (Han et al., 2012), and chickens (Zhang et al., 2015) no variation was found in gene expression. On the other hand, changes in gene expression were found in Japanese quails, which present greater ASIP expression in individuals with brown plumage and larger MC1R expression in black plumage organisms (Zhang et al., 2013a). In melanic chickens, an increase in ASIP was detected (Zhang et al., 2015), while dark specimens of the Italian lizard *Podarcis siculus* presented a high amount of MC1R mRNA (Fulgione et al., 2015).

Some of these gene expression studies associated with melanism have been carried out based on different stages of development. In vertebrates, diverse processes may contribute to variation in melanin type and density. During vertebrate embryogenesis, the dorsal neural tube produces neural crest cells, and some differentiate into melanoblasts (precursor cells of melanocytes), which migrate through the body. Melanoblasts normally localise in the epidermis or hair follicles where they differentiate into melanocytes which are in charge of producing pigment; melanosomes package the pigment and are then transferred to keratinocytes of hair or epidermal cells. Therefore, it is possible that genes involved in melanism or colour patterning act early in development and are involved in melanocyte differentiation, development, and migration (Hoekstra, 2006). Thus, developmental timing could play an important role in colour patterning (Bard, 1977). Most progress in understanding patterning has been made in mice, uncovering genes (ASIP) which determine differences in dorsal-ventral pigmentation (Bultman et al., 1992; Miller et al., 1993; Millar et al., 1995). In Japanese quail embryos, a high expression of MC1R activator agonist was found in hyperpigmented individuals during the latest stages of development (Gluckman & Mundy, 2017; Li et al., 2018). Further, a greater expression of MC1R was found in Silky Fowl, which presents hyperpigmentation in several internal tissues (Li et al., 2011); and in *Astyanax* cavefish, where a variable pattern of pigmentation gene expression of MC1R across development stages was described, but only a significantly different morphotype-specific expression at the adult stage was observed (Stahl & Gross, 2015).

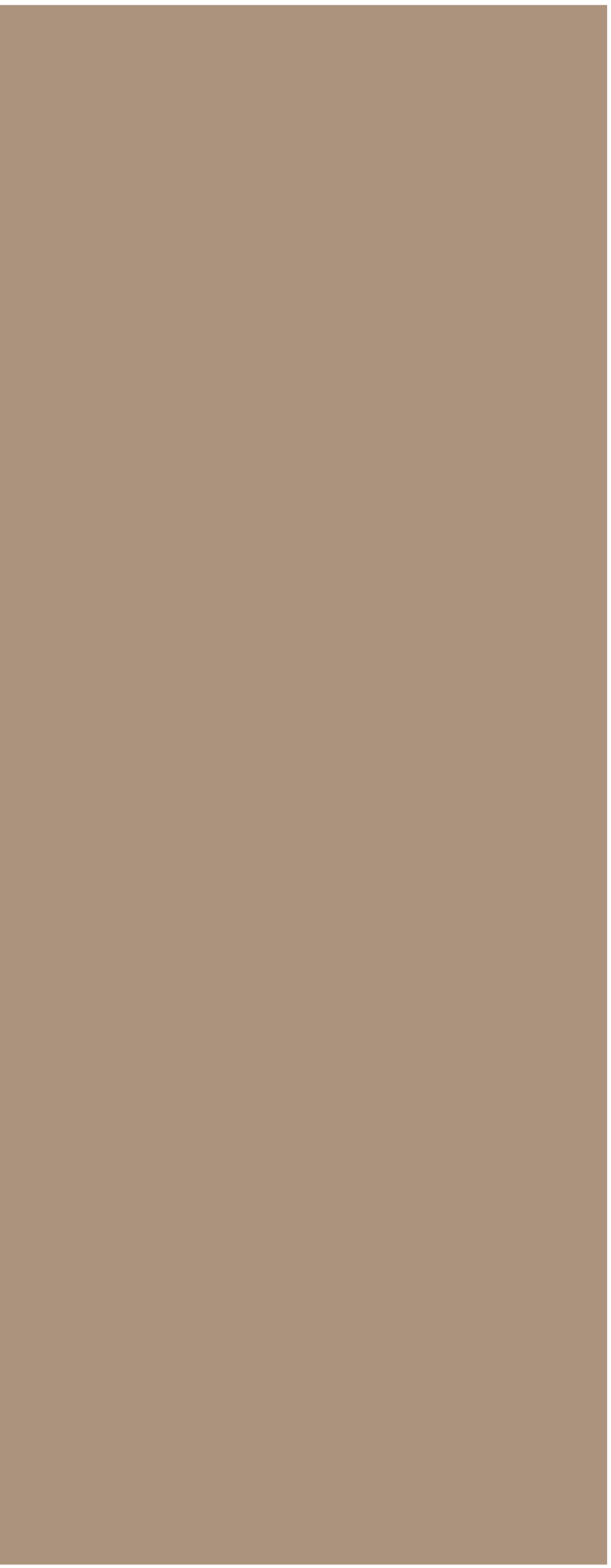
1.3.3. Melanism in insular populations

The presence of similar traits across different organisms which inhabited islands, such as the loss of flight in birds or changes in body size suggest that other processes, rather than genetic drift, control the convergent evolution of these characteristics (Uy & Vargas-Castro, 2015). One of these characteristics that appears regularly in island populations is melanism. We can find examples of melanism on islands in different species of birds: *Coereba flaveola* (Theron et al., 2001), *Malurus leucopterus* (Doucet et al., 2004), *Myzomela* spp. (Mayr & Diamond, 2001), *Rhipidura fuliginosa* (Atkinson & Briskie, 2007), *Turdus poliocephalus* (Jones & Kennedy, 2008); reptiles: *Elaphe quadrivirgata* (Tanaka, 2007), *Nerodia sipedon* (King, 1993), *Podarcis lilfordi* (Buades et al., 2013), *Thamnophis sirtalis* (Bittner & King, 2003);

spiders: *Nephila maculata* (Tso et al., 2002); and insects: *Oedaleus senegalensis* (Ritchie, 1978), *Philaenus spumarius* (Berry & Willmer, 1986; Brakefield, 1990; Halkka et al., 2001). As indicated above, melanism patterns might be adaptive (Garcia et al., 2003; Escudero et al., 2016) or non-adaptive to environmental diversity (Alho et al., 2010; Fulgione et al., 2015). However, in most cases, the determining factors of the appearance of melanism as an evolutionary trait are poorly understood.

2

Aims



The main goal of this thesis was to study adaptive processes in *Podarcis* genus from different genetic approaches and better understand how different evolutionary processes have configured patterns of divergence at the genomic and phenotypic level.

To achieve this main objective, specific aims were defined:

1. Phylogenetic and phylogeographic characterization of the *P. hispanicus* complex in the southeast (SE) region of the Iberian Peninsula by means of a multilocus analysis with extensive sampling of the different *Podarcis* lineages.

2. Analysis of the *Podarcis* population from the Columbretes Islands in order to establish their population structure and explore their evolutionary origin and their relationship with other mainland forms.

3. Assess, at fine-scale level, the intra-specific variability of the whole distribution range of *P. lilfordi* in order to evaluate the effect of different evolutionary factors that have shaped the divergence of these insular populations and their consequences in conservation policies; by the use of morphological, ecological, and genetic markers, as well as genomic analyses.

4. Through genome-wide analysis of *P. lilfordi* populations, discover region candidates to be under selection and evaluate whether the characteristic traits of these populations are the result of stochastic effects of genetic drift or are the result of natural selection. Evaluate the association between genomic divergence and phenotypical (melanism) and environmental (biotic capacity, number of vascular plants, predation, human pressure, presence of rats and/or seagulls) variables characteristic of the different Balearic lizard populations.

5. Explore the relationship between MC1R gene expression and the phenotypical feature of melanism in *P. lilfordi*, by carrying out these analyses in different stages of development (embryonic and adult stages) in melanic and non-melanic populations.

3

Material & Methods

3.1. Iberian *Podarcis* complex

3.1.1. Samples

Lizards were caught by careful noosing with specific permits delivered by the competent authority in each locality. Light finger pressure on the tail tips caused tail autotomy. We were careful to remove only a minimal amount of tail tissue (not exceeding 8 mm) and lizards were immediately released at their points of capture, without sampling if they showed any signs of stress.

In total, 162 individuals from the Iberian Peninsula and North African region were captured. Tail tip sample was stored in 100% ethanol at 4°C. Samples and their locations are described in Figure 17 and Table 1.

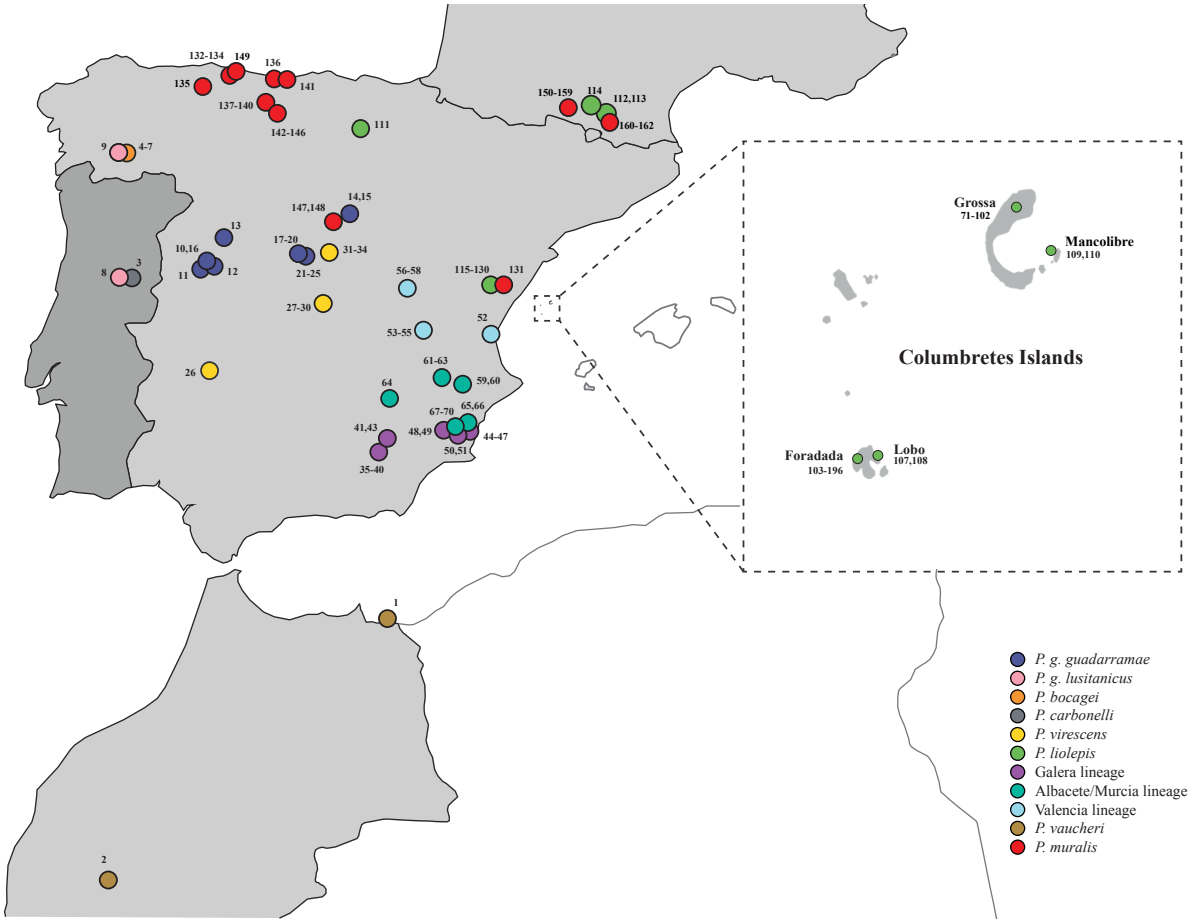


Figure 17. Locations of all *Podarcis* samples from Iberian Peninsula and North Africa used in this study. Numbers correspond to those in Table 1.

Table 1. Species or subspecies assignation, code number, geographic location and GenBank Accession Number for all the samples from the Iberian *Podarcis* complex used in this study and the *Podarcis* from Balearic Island used as outgroup. NADH=ND1+ND2+tRNAs ($tRNA_{Ile}$, $tRNA_{Gln}$, and $tRNA_{Met}$).

	Species	Code	Country	Locality	Lat	Long	12S	CR	CYTB	NADH	RAG1	MC1R	KIAA2018	APOBE28
1	*P. vaucheri*	Vau01	Spain	Chafarinas Islands	35.182	-2.430	KT030729.1	KT030727.1	KT030723.1	KT030725.1	MN651838	MN651708		
2	*P. vaucheri*	Vau02	Morocco	Oukaïmeden	31.202	-7.858	KT030730.1	KT030728.1	KT030724.1	KT030726.1		MN651709		
3	*P. carbonelli*	CAR01	Portugal	Lagoa Comprida	40.365	-7.647	KT030681.1	KT030679.1	KT030675.1	KT030677.1	MN651839	MN651710		
4	*P. bocagei*	BN1	Spain	La Baña	42.289	-6.670					+			
5	*P. bocagei*	BN3	Spain	La Baña	42.289	-6.670	MN649217	MN651572	MN651598	MN651659	MN651840	MN651711		
6	*P. bocagei*	BN5	Spain	La Baña	42.289	-6.670	MN649217	MN651572	MN651598	MN651660	MN651841	MN651712		
7	*P. bocagei*	HN2	Spain	La Baña	42.289	-6.670					+	+		
8	*P. g. lusitanicus*	CAR02	Portugal	Lagoa Comprida	40.365	-7.647	KT030682.1	KT030680.1	KT030676.1	KT030678.1	MN651842	MN651713		
9	*P. g. lusitanicus*	HN1	Spain	La Baña	42.289	-6.670	MN649218	MN651573	MN651599	MN651661	MN651843	MN651714		
10	*P. g. guadarramae*	HE4	Spain	Escorial de la Sierra, Salamanca	40.614	-5.951	MN649219	MN651574	MN651600	MN651662	MN651844	MN651715		
11	*P. g. guadarramae*	HM3	Spain	Mogarraz, Salamanca	40.501	-6.063	KT030705.1	MN651575	MN651601	MN651663	MN651845	MN651716		
12	*P. g. guadarramae*	HS1	Spain	Los Santos, Salamanca	40.545	-5.797	MN649220	KT030700.1	MN651602	MN651664	MN651846	MN651717		
13	*P. g. guadarramae*	HC1	Spain	Cabrerizos, Salamanca	40.979	-5.608	KT030705.1	KT030700.1	KT030688.1	KT030694.1	MN651847	MN651718		
14	*P. g. guadarramae*	HT1	Spain	Tiermes, Soria	41.336	-3.153	MN649221	MN651576	MN651603	MN651665	MN651848	MN651719		
15	*P. g. guadarramae*	HP2	Spain	Pedro, Soria	41.317	-3.194	MN649221	MN651576	MN651603	MN651665	MN651849	MN651720		
16	*P. g. guadarramae*	His3	Spain	Escorial de la sierra, Salamanca	40.614	-5.951	MN649219	MN651574	MN651600	MN651662	MN651850	MN651721		
17	*P. g. guadarramae*	HGF1	Spain	Fuenfria, Madrid	40.741	-4.066	MN649221	MN651574	MN651604	MN651665	MN651851	MN651722		
18	*P. g. guadarramae*	HGF2	Spain	Fuenfria, Madrid	40.741	-4.066	MN649221	MN651576	MN651605	MN651665	MN651852	MN651723		
19	*P. g. guadarramae*	HGF5	Spain	Fuenfria, Madrid	40.741	-4.066	MN649221	MN651576	MN651606	MN651666	MN651853	MN651724		
20	*P. g. guadarramae*	HGF	Spain	Fuenfria, Madrid	40.741	-4.066	MN649221	MN651577	MN651605	MN651665	MN651854	MN651725		
21	*P. g. guadarramae*	HGG1	Spain	Navacerrada, Madrid	40.722	-4.024	MN649221	MN651576	MN651607	MN651665	MN651855	MN651726		
22	*P. g. guadarramae*	HGG2	Spain	Navacerrada, Madrid	40.722	-4.024	MN649221	MN651576	MN651608	MN651667	MN651856	MN651727		
23	*P. g. guadarramae*	HGG3	Spain	Navacerrada, Madrid	40.722	-4.024	MN649221	MN651577	MN651605	MN651665	MN651857	MN651728		
24	*P. g. guadarramae*	HGG4	Spain	Navacerrada, Madrid	40.722	-4.024	MN649221	MN651576	MN651605	MN651668	MN651858	MN651729		
25	*P. g. guadarramae*	HGG5	Spain	Navacerrada, Madrid	40.722	-4.024	MN649221	MN651576	MN651609	MN651669	MN651859	MN651730		
26	*P. virescens*	HD1	Spain	Don Benito, Badajoz	38.956	-5.864	KT030704.1	KT030699.1	KT030687.1	KT060693.1	MN651860	MN651731		
27	*P. virescens*	HVA1	Spain	Aranjuez, Madrid	39.995	-3.672	MN649222	MN651578	MN651610	MN651670	MN651861	MN651732		
28	*P. virescens*	HVA2	Spain	Aranjuez, Madrid	39.995	-3.672	MN649223	MN651578	MN651610	MN651670	MN651862	MN651733		
29	*P. virescens*	HVA3	Spain	Aranjuez, Madrid	39.995	-3.672	MN649223	MN651578	MN651610	MN651670	MN651863	MN651734		
30	*P. virescens*	HVA4	Spain	Aranjuez, Madrid	39.995	-3.672	MN649223	MN651578	MN651610	MN651670	MN651864	MN651735		
31	*P. virescens*	HVP2	Spain	Pedrezuela, Madrid	40.750	-3.612	MN649224	MN651578	MN651611	MN651671	MN651865	MN651736		
32	*P. virescens*	HVP3	Spain	Pedrezuela, Madrid	40.750	-3.612	MN649224	MN651578	MN651612	MN651670	MN651866	MN651737		
33	*P. virescens*	HVP4	Spain	Pedrezuela, Madrid	40.750	-3.612	MN649224	MN651578	MN651612	MN651670	MN651867	MN651738		
34	*P. virescens*	HVP5	Spain	Pedrezuela, Madrid	40.750	-3.612	MN649224	MN651578	MN651612	MN651670	MN651868	MN651739		
35	Galera lineage	Gale1	Spain	Galera, Granada	37.739	-2.563	MN649225	MN651579	MN651613	MN651672	MN651869	MN651740		
36	Galera lineage	Gale2	Spain	Galera, Granada	37.739	-2.563					+	+		
37	Galera lineage	Gale3	Spain	Galera, Granada	37.739	-2.563	MN649225	MN651579	MN651613	MN651672	MN651870	MN651741		
38	Galera lineage	Gale5	Spain	Galera, Granada	37.739	-2.563	MN649225	MN651579	MN651613	MN651672	MN651871	MN651742		
39	Galera lineage	Gale6	Spain	Galera, Granada	37.739	-2.563						+		
40	Galera lineage	Gale7	Spain	Galera, Granada	37.739	-2.563						+		
41	Galera lineage	Df1	Spain	Puebla de Don Fadrique, Granada	37.911	-2.398	MN649225	MN651580	MN651613	MN651672	MN651872	MN651743		
42	Galera lineage	Df2	Spain	Puebla de Don Fadrique, Granada	37.911	-2.398						+		
43	Galera lineage	Df3	Spain	Puebla de Don Fadrique, Granada	37.911	-2.398	MN649225	MN651581	MN651613	MN651672		MN651744		
44	Galera lineage	Ph16	Spain	Orihuela, Alicante	38.085	-0.948	MN649225	MN651580	MN651614	MN651673	MN651873	MN651745		
45	Galera lineage	Ph21	Spain	Embalse de la Pedrera, Orihuela	38.032	-0.870	MN649225	MN651580	MN651615	MN651674		MN651746		

46	Galera lineage	Ph22	Spain	Embalse de la Pedrera, Orihuela	38.032	-0.870	MN649226	MN651580	MN651614	MN651673		MN651747		
47	Galera lineage	Ph23	Spain	Embalse de la Pedrera, Orihuela	38.032	-0.870						+		
48	Galera lineage	Ro1	Spain	Embalse de los Rodeos, Murcia	38.043	-1.295	MN649225	MN651580	MN651616	MN651673	MN651874	MN651748		
49	Galera lineage	Ro2	Spain	Embalse de los Rodeos, Murcia	38.043	-1.295	MN649225	MN651580	MN651617	MN651673		MN651749		
50	Galera lineage	AB8	Spain	Las Canteras, Murcia	37.634	-1.041	MN649225	MN651580	MN651618	MN651675	MN651875	MN651750		
51	Galera lineage	AB9	Spain	Las Canteras, Murcia	37.634	-1.041	MN649225	MN651580	MN651614	MN651676	MN651876	MN651751		
52	Valencia lineage	HB2	Spain	Burjassot, Valencia	39.511	-0.414	KT030701.1	KT030695.1	MN651619	KT030689.1	MN651877	MN651752		
53	Valencia lineage	PHP3	Spain	Puebla Salvador, Cuenca	39.563	-1.675	MN649227	MN651582	MN651620	MN651677	MN651878			
54	Valencia lineage	PHP4	Spain	Puebla Salvador, Cuenca	39.563	-1.675	MN649227	MN651582	MN651621	MN651678	MN651879	MN651753		
55	Valencia lineage	PHP5	Spain	Puebla Salvador, Cuenca	39.563	-1.675					+	+		
56	Valencia lineage	PHCE2	Spain	Ciudad Encantada, Cuenca	40.208	-2.011	KT030701.1	MN651582	MN651622	KT030689.1	MN651880	MN651754		
57	Valencia lineage	PHCE4	Spain	Ciudad Encantada, Cuenca	40.208	-2.011	KT030701.1	MN651582	MN651623	MN651679	MN651881	MN651755		
58	Valencia lineage	PHCE5	Spain	Ciudad Encantada, Cuenca	40.208	-2.011					+	+		
59	Albacete/Murcia lineage	Alb1	Spain	Sierra de la Oliva, Albacete	38.765	-0.971	MN649228	MN651583	MN651624	MN651680		MN651756		
60	Albacete/Murcia lineage	Alb2	Spain	Sierra de la Oliva, Albacete	38.765	-0.971	MN649228	MN651583	MN651624	MN651680		MN651757		
61	Albacete/Murcia lineage	Alb3	Spain	Montealegre del Castillo, Albacete	38.826	-1.341	MN649228	MN651583	MN651625	MN651680	MN651882	MN651758		
62	Albacete/Murcia lineage	Alb4	Spain	Montealegre del Castillo, Albacete	38.826	-1.341	MN649228	MN651584	MN651626	MN651680	MN651883	MN651759		
63	Albacete/Murcia lineage	Alb5	Spain	Montealegre del Castillo, Albacete	38.826	-1.341						+		
64	Albacete/Murcia lineage	Alb6	Spain	Cañada del Provencio, Albacete	38.518	-2.353	MN649228	MN651583	MN651627	MN651680	MN651884	MN651760		
65	Albacete/Murcia lineage	Seg1	Spain	Sierra Callosa del Segura, Alicante	38.123	-0.895						+		
66	Albacete/Murcia lineage	Seg2	Spain	Sierra Callosa del Segura, Alicante	38.123	-0.895	MN649228	MN651583	MN651628	MN651680	MN651885	MN651761		
67	Albacete/Murcia lineage	Cmp1	Spain	Laderas del Campillo, Murcia	38.060	-1.092	MN649228	MN651583	MN651629	MN651681	MN651886	MN651762		
68	Albacete/Murcia lineage	Cmp2	Spain	Laderas del Campillo, Murcia	38.060	-1.092	MN649228	MN651583			MN651887	MN651763		
69	Albacete/Murcia lineage	Cmp3	Spain	Laderas del Campillo, Murcia	38.060	-1.092						+		
70	Albacete/Murcia lineage	Cmp4	Spain	Laderas del Campillo, Murcia	38.060	-1.092	MN649228	MN651583	MN651629	MN651681		MN651764		
71	*P. liolepis*	AB0	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651630	MN651682	MN651888	MN651765		
72	*P. liolepis*	AB1	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651631	MN651683	MN651889	MN651766	+	+
73	*P. liolepis*	AB2	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683	MN651890	MN651767		
74	*P. liolepis*	AB3	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683	MN651891	MN651768	+	+
75	*P. liolepis*	AB4	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651684	MN651892	MN651769	+	+
76	*P. liolepis*	AB5	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651685	MN651893	MN651770	+	+
77	*P. liolepis*	AB6	Spain	Grossa, Columbretes Islands	39.898	0.686						+		
78	*P. liolepis*	AB7	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651633	MN651684	MN651894	MN651771	+	+
79	*P. liolepis*	AB10	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683	MN651895	MN651772		
80	*P. liolepis*	AB11	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651631	MN651683	MN651896	MN651773		
81	*P. liolepis*	AB12	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651634		MN651897	MN651774		
82	*P. liolepis*	AB13	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683	MN651898	MN651775		+
83	*P. liolepis*	AB14	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683		+		
84	*P. liolepis*	AB22	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651630	MN651682	MN651899	MN651776		
85	*P. liolepis*	AB32	Spain	Grossa, Columbretes Islands	39.898	0.686						+		
86	*P. liolepis*	AB33	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683	MN651900	MN651777	+	+
87	*P. liolepis*	AB44	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651631	MN651683	MN651901	MN651778	+	+
88	*P. liolepis*	AB52	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683	MN651902	MN651779		
89	*P. liolepis*	AB55	Spain	Grossa, Columbretes Islands	39.898	0.686		MN651585	MN651632	MN651683	MN651903	MN651780		
90	*P. liolepis*	AB62	Spain	Grossa, Columbretes Islands	39.898	0.686						+		
91	*P. liolepis*	AB66	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683		+		

92	*P. liolepis*	AB72	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683		+			
93	*P. liolepis*	AB77	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1		MN651632		MN651904	MN651781			
94	*P. liolepis*	AB88	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651684	MN651905	MN651782			
95	*P. liolepis*	AB001	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683	MN651906	MN651783			
96	*P. liolepis*	AB002	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683		+			
97	*P. liolepis*	AB007	Spain	Grossa, Columbretes Islands	39.898	0.686					+				
98	*P. liolepis*	AB008	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651631	MN651683		+			
99	*P. liolepis*	AB009	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651633	MN651683	MN651907	MN651784			
100	*P. liolepis*	AB012	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683	+				
101	*P. liolepis*	AB017	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651635	MN651683	+				
102	*P. liolepis*	AB022	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683	+				
103	*P. liolepis*	AF1	Spain	Foradada, Columbretes Islands	39.875	0.671	KT030702.1	MN651585	MN651636		MN651908	MN651785			
104	*P. liolepis*	AF2	Spain	Foradada, Columbretes Islands	39.875	0.671	KT030702.1	MN651585	MN651636	MN651682	MN651909	MN651786	+		
105	*P. liolepis*	AF3	Spain	Foradada, Columbretes Islands	39.875	0.671					+				
106	*P. liolepis*	AF4	Spain	Foradada, Columbretes Islands	39.875	0.671					+				
107	*P. liolepis*	AO2	Spain	Lobo, Columbretes Islands	39.875	0.672	KT030702.1	MN651585	MN651636		MN651910	MN651787			
108	*P. liolepis*	AO3	Spain	Lobo, Columbretes Islands	39.875	0.672	KT030702.1	MN651585	MN651636	MN651682	MN651911	MN651788	+	+	
109	*P. liolepis*	AM1	Spain	Mancolibre, Columbretes Islands	39.895	0.690	KT030702.1	MN651585	MN651632		MN651912	MN651789			
110	*P. liolepis*	AM2	Spain	Mancolibre, Columbretes Islands	39.895	0.690	KT030702.1	MN651585	MN651632	MN651686	MN651913	MN651790	+	+	
111	*P. liolepis*	HL1	Spain	Sazajarra, Rioja	42.588	-2.961	KT030703.1	KT030698.1	MN651637	KT030692.1	MN651914	MN651791	+	+	
112	*P. liolepis*	PL3	France	Pêch de Foix	42.963	1.623	MN649229	MN651586	MN651638	MN651687	MN651915	MN651792	+	+	
113	*P. liolepis*	PL36	France	Pêch de Foix	42.963	1.623							+	+	
114	*P. liolepis*	PL60	France	Vaychis	42.748	1.840	KT030702.1	MN651587	MN651639	MN651682	MN651916	MN651793	+	+	
115	*P. liolepis*	HF9	Spain	Peñagolosa, Castellón	40.245	-0.376	KT030702.1	KT030697.1	KT030685.1	KT030691.1	MN651917	MN651794	+	+	
116	*P. liolepis*	HJ2	Spain	Peñagolosa, Castellón	40.245	-0.376	KT030702.1	MN651587	KT030684.1	KT030690.1	MN651918	MN651795	+	+	
117	*P. liolepis*	HJ3	Spain	Peñagolosa, Castellón	40.245	-0.376	KT030702.1	MN651587	MN651640	MN651688	MN651919	MN651796	+	+	
118	*P. liolepis*	HJ4	Spain	Peñagolosa, Castellón	40.245	-0.376	KT030702.1	MN651587	MN651641	MN651689	MN651920	MN651797	+	+	
119	*P. liolepis*	HPC8	Spain	Peñagolosa, Castellón	40.245	-0.376	KT030702.1	MN651587	MN651640	MN651690	MN651921	MN651798		+	
120	*P. liolepis*	MPC5	Spain	Peñagolosa, Castellón	40.245	-0.376	KT030702.1	MN651587	KT030707.1	KT030709.1	MN651922	MN651799	+	+	
121	*P. liolepis*	MPC6	Spain	Peñagolosa, Castellón	40.245	-0.376	KT030702.1	MN651588	MN651642	MN651690	MN651923	MN651800	+	+	
122	*P. liolepis*	HP118	Spain	Peñagolosa, Castellón	40.245	-0.376	KT030702.1	MN651587	MN651643	MN651691	MN651924	MN651801			
123	*P. liolepis*	HP218	Spain	Peñagolosa, Castellón	40.245	-0.376	KT030702.1	MN651587	MN651640	MN651692	MN651925	MN651802			
124	*P. liolepis*	HP3	Spain	Peñagolosa, Castellón	40.245	-0.376	MN649230	MN651587	MN651644	MN651693	MN651926	MN651803			
125	*P. liolepis*	HP4	Spain	Peñagolosa, Castellón	40.245	-0.376	KT030702.1	MN651587	MN651645	MN651694	MN651927	MN651804			
126	*P. liolepis*	HP5	Spain	Peñagolosa, Castellón	40.245	-0.376	KT030702.1	MN651589	MN651646	KT030690.1	MN651928	MN651805			
127	*P. liolepis*	HP8	Spain	Peñagolosa, Castellón	40.245	-0.376	KT030702.1	MN651585	MN651642	MN651690	MN651929	MN651806			
128	*P. liolepis*	HP9	Spain	Peñagolosa, Castellón	40.245	-0.376	KT030702.1	MN651587	MN651647	MN651690	MN651930	MN651807			
129	*P. liolepis*	HP10	Spain	Peñagolosa, Castellón	40.245	-0.376	KT030702.1	MN651587	MN651641	KT030709.1	MN651931	MN651808			
130	*P. liolepis*	HP11	Spain	Peñagolosa, Castellón	40.245	-0.376	KT030702.1	MN651590	MN651648	KT030690.1	MN651932	MN651809			
131	*P. muralis*	Mpfn7	Spain	Peñagolosa, Castellón	40.245	-0.376	MN649231	MN651591	MN651649	MN651695	MN651933	MN651810			
132	*P. muralis*	MGV1	Spain	Villaviciosa, Asturias	43.482	-5.433	MN649232	MN651592	MN651650	MN651696	MN651934	MN651811			
133	*P. muralis*	MLL1	Spain	Villaviciosa, Asturias	43.482	-5.433	MN649232	MN651592	MN651650	MN651697	MN651935	MN651812	+	+	
134	*P. muralis*	MLL2	Spain	Villaviciosa, Asturias	43.482	-5.433	MN649232	MN651592	MN651650	MN651698	MN651936	MN651813			
135	*P. muralis*	MOP1	Spain	Proaza, Asturias	43.258	-6.006	MN649232	MN651593	MN651651	MN651699	MN651937	MN651814			
136	*P. muralis*	MPN1	Spain	Pendueles, Asturias	43.397	-4.633	MN649232	MN651593	MN651651	MN651699	MN651938	MN651815			
137	*P. muralis*	MPG2	Spain	Pto San Glorio, Cantabria	43.067	-4.766	MN649232	MN651593	MN651651	MN651699	MN651939	MN651816	+	+	

138	*P. muralis*	MPG3	Spain	Pto San Glorio, Cantabria	43.067	-4.766	MN649232	MN651593	MN651652	MN651699	MN651940	MN651817		
139	*P. muralis*	MPG4	Spain	Pto San Glorio, Cantabria	43.458	-3.814	MN649232	MN651593	MN651653	MN651699	MN651941	MN651818		
140	*P. muralis*	MPG5	Spain	Pto San Glorio, Cantabria	43.458	-3.814	MN649232	MN651593	MN651651	MN651699	MN651942	MN651819		
141	*P. muralis*	MVB1	Spain	San Vicente de la Barquera, Cantabria	43.381	-4.399	MN649232	MN651593	MN651651	MN651700	MN651943	MN651820		
142	*P. muralis*	MSP1	Spain	Cervera, Castilla y León	42.867	-4.500	MN649232	MN651593	MN651651	MN651701	MN651944	MN651821	+	+
143	*P. muralis*	MSP2	Spain	Cervera, Castilla y León	42.867	-4.500	MN649232	MN651593	MN651652	MN651699	MN651945	MN651822		
144	*P. muralis*	MSP3	Spain	Cervera, Castilla y León	42.867	-4.500	MN649232	MN651593	MN651652	MN651699	MN651946	MN651823		
145	*P. muralis*	MSP4	Spain	Cervera, Castilla y León	42.867	-4.500	MN649232	MN651593	MN651652	MN651699	MN651947	MN651824		
146	*P. muralis*	MSP5	Spain	Cervera, Castilla y León	42.867	-4.500	MN649232	MN651593	MN651651	MN651699	MN651948	MN651825		
147	*P. muralis*	MLP1	Spain	La Pinilla, Castilla y León	41.222	-3.479	KT030712.1	KT030710.1	KT030706.1	KT030708.1	MN651949	MN651826	+	+
148	*P. muralis*	MLP3	Spain	La Pinilla, Castilla y León	41.222	-3.479	KT030712.1	KT030710.1	KT030706.1	MN651702				
149	*P. muralis*	MSS1	Spain	Selorio, Asturias	43.515	-5.362	MN649232	MN651593	MN651651	MN651703	MN651950	MN651827		
150	*P. muralis*	MP2	France	Moulis	42.960	1.092	MN649233	MN651594	MN651654	MN651704	MN651951	MN651828		
151	*P. muralis*	MP3	France	Moulis	42.960	1.092	MN649234	MN651595	MN651655	MN651705	MN651952	MN651829		
152	*P. muralis*	MP4	France	Moulis	42.960	1.092	MN649234	MN651595	MN651655	MN651705	MN651953	MN651830		
153	*P. muralis*	MP5	France	Moulis	42.960	1.092	MN649233	MN651594	MN651654	MN651704	MN651954	MN651831		
154	*P. muralis*	MP6	France	Moulis	42.960	1.092	MN649234	MN651595	MN651655	MN651706				
155	*P. muralis*	MP7	France	Moulis	42.960	1.092	MN649235	MN651596	MN651656	MN651704	MN651955	MN651832		
156	*P. muralis*	MP8	France	Moulis	42.960	1.092	MN649235	MN651594	MN651657	MN651704				
157	*P. muralis*	MP9	France	Moulis	42.960	1.092	MN649233	MN651594	MN651654	MN651704	MN651956	MN651833		
158	*P. muralis*	MP10	France	Moulis	42.960	1.092	MN649234	MN651595	MN651655	MN651705	MN651957	MN651834		
159	*P. muralis*	MP11	France	Moulis	42.960	1.092	MN649235	MN651594	MN651656	MN651704	MN651958	MN651835		
160	*P. muralis*	MP13	France	Vaychis	42.748	1.840	MN649234	MN651595	MN651658	MN651705	MN651959	MN651836		
161	*P. muralis*	MP14	France	Vaychis	42.748	1.840	MN649235	MN651597	MN651656	MN651707	MN651960	MN651837		
162	*P. muralis*	MP15	France	Vaychis	42.748	1.840	MN649235	MN651597	MN651656	MN651707				
	Outgroup*													
	P. lilfordi	D22	Spain	Dragonera, Balearic Islands	39.584	2.320	EF694761.1	EF694773.1	KC623944.1	EU006730.1	MN651964	JX126631.1		
	P. lilfordi	Cr1	Spain	Es Colomer, Balearic Islands	39.945	3.131	EF694760.1	EF694775.1	EF694801.1 / EF990524.1	EU006737.1				
	P. lilfordi	Cp1	Spain	Cabrera harbour, Balearic Islands	39.151	2.934	EF694764.1	EF694780.1	KF003361.1 /EF990531.1	EU006743.1	MN651963	JX126652.1		
	P. lilfordi	Cf	Spain	Cabrera lighthouse, Balearic Islands	39.129	2.921					MN651961	JX126649.1		
	P. lilfordi	X3	Spain	Estel Xapat, Balearic Islands	39.125	2.939	EF694765.1	EF694785.1	EF694807.1 / EF990539.1	EU006753.1				
	P. lilfordi	AG1	Spain	Addaia Gran, Balearic Islands	40.016	4.210	EF694766.1	EF694786.1	EF694809.1 / EF694826.1	EU006756.1				
	P. lilfordi	CI	Spain	Binicodrell, Balearic Islands	39.916	4.030					MN651962	JX126640.1		
	P. pityusensis	Alg1	Spain	Alga, Balearic Islands	38.977	1.532	EF694768.1	EF694794.1	JX852075.1	JX852119.1				
	P. pityusensis	Eiv1	Spain	Ibiza, Balearic Islands	38.907	1.421	EF694768.1	EF694794.1	JX852050.1	JX852121.1	MN651965	JX126670.1		
	P. pityusensis	Fort4	Spain	Formentera, Balearic Islands	38.753	1.436	EF694768.1	EF694794.1	JX852075.1	JX852121.1	MN651966	JX126678.1		
	P. pityusensis	Fx	Spain	Formentera, Balearic Islands	38.706	1.429					MN651967	JX126678.1		

***Brown et al., 2008; Terrasa et al., 2009a; Buades et al., 2013; Kapli et al., 2013; Rodriguez et al., 2013**

3.1.2. DNA extraction, amplification, sequencing, and dataset assembly

A standard phenol-chloroform protocol was used for DNA extraction (González et al., 1996; with modifications). Before treatment, alcohol samples were washed in 10 ml distilled water for 30 min. DNA was isolated from fine slices of tail tissue and homogenised in 4 ml of lysis buffer (10 mM Tris-HCl, 50 mM KCl, 25 mM $MgCl_2$, 0.45% Igepal CA-630, 0.45% Tween 20, pH 8.0). Detergents present in buffer and the mechanical action exercised by a homogeniser disaggregated tissue samples. Then, 500 µl of 20 % SDS and 10 µl Proteinase K (20 mg/ml) was added for cell lysis. The mixture was incubated at 55°C for 4 h or at 37°C overnight (o/n). Proteins and lipids were eliminated by three consecutive centrifugations for 10 min at 1,800 g in a Centromix Centrifuge (JP Selecta, Spain). In the first centrifugation, the same volume of phenol and sample was added, the aqueous phase was recovered and the same volume of phenol:chlorofrom:isoamyl alcohol (25:24:1) was mixed for the second centrifugation. Ultimately, to recovered the aqueous phase, the same volume of chloroform:isoamyl alcohol (24:1) was added. The final supernatant was subjected to a precipitation process to achieve pure DNA. Per each 5 ml of sample, 400 µl of 5 M NaCl was added, absolute ethanol was also added until it reached a total volume of 8 ml. Samples were stored at -20°C o/n. A centrifugation at 3,200 g for 20 min was carried out and the aqueous phase was removed. Three millilitres of 70% ethanol was added, and samples were centrifuged for 10 min at 3,200 g. The aqueous phase was eliminated, and samples were dried in a vacuum pump. DNA pellet was resuspended in 100 µl of Milli-Q water and quantified with NanoVue Plus™ (GE healthcare, Chicago EUA). DNA with a concentration of 80 ng/µl was used as a template for subsequent PCR amplifications.

The following five non-overlapping mtDNA fragments were obtained: i) partial 12S rRNA (360 bp), ii) partial control region (CR) (476 bp), iii) a short partial cytochrome b (CYTB) fragment (306 bp), iv) a long partial CYTB fragment (483 bp), and v) two partial subunits of the NADH dehydrogenase gene and associated tRNAs (referred to as ND1 (48 bp), ND2 (415 bp), $tRNA_{Ile}$, $tRNA_{Gln}$, and $tRNA_{Met}$ (213 bp)). Four partial nuclear genes were amplified and sequenced: (i) melanocortin 1 receptor gene (MC1R) (663 bp), ii) recombination activating gene 1 (RAG1) (939 bp), iii) apolipoprotein B (APOBE28) (489 bp) and iv) transcription factor gene (KIAA2018) (623 bp).

Mitochondrial and nuclear fragments were amplified by PCR with thermocyclers GeneAmp 2700 (Applied Biosystems) with primers and amplification conditions showed in Table 2. PCR reaction volume was 25 µl consisting of buffer 10X (2.5 µl), 50 mM MgCl2 (1.75 µl), 0.2 mM of each dNTPs, primers with 20 mM, 1U of DNA polymerase BIOTAQ (Biotools), and 80 ng of DNA sample. A negative control was included in each reaction. UV MiniBis BioImaging System (DNR Bio-Imaging Systems, Germany) checked positive amplification by means of a 1% agarose gel using ethidium bromide. Purification of amplified fragments was performed by MSB Spin PCRapace kit (Invitek, Germany) and while quantifications and quality ratio analysis were determined using the spectrophotometer NanoVue™ Plus spectrophotometer (GE HealthCare, UK).

Both strands of the PCR products were sequenced using the Sanger method (Sanger et al., 1977) in an automated ABI 3130 sequencer (Applied Biosystems, Foster City, CA, USA) using a BigDye® Terminator v. 3.1 Cycle sequencing kit (Applied Biosystems). Sequencing conditions were 96ºC for 3 min, 25 cycles of 96ºC for 10 sec, 50ºC for 5 sec, and 60ºC for 4 min. Sequences obtained were edited using CodonCode Aligner software (CodonCode Corporation, Dedham, MA, USA).

Mitochondrial DNA genes and the four nuclear fragments were aligned in the MAFFT v7.423 online server (Katoh & Toh, 2008) using the iterative refinement method (FFT-NS-i). For the protein-coding genes, alignments were verified by translating nucleotide sequences to amino acids using MEGA7 (Kumar et al., 2016). Mitochondrial DNA fragments were concatenated in a 2,301 bp unique alignment and nuclear data were phased using the PHASE algorithm (Stephens et al., 2001) using DnaSP v.6 (Librado & Rozas, 2009). This algorithm uses a coalescent-based Bayesian method to infer the haplotypes from sequences with heterozygotic positions. Thus, from a sequence with heterozygous positions, we obtain two sequences with all homozygous positions.

Table 2. Sequences of the nine pairs of primers of mtDNA and nuclear gene fragments amplified in this study and the PCR conditions used in each case.

Gene	Primer sequences	Reference	PCR conditions		
12S					
L1091 H1478	5'-AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT-3' 5'-TGACTGCAGAGGGTGACGGGCGGTGTGT-3'	Kocher et al. (1989)	96°C 94°C 50°C 72°C 72°C	5' 1' 1' 1' 5'	30 cycles
CR					
L15022 H00292	5'-TACCCTTGCTCATAGCATAACTG-3' 5'-GTCTTGTTGACTGTAATTAACCGATA-3'	modified from Brehm et al. (2003)	96°C 94°C 50°C 72°C 72°C	5' 1' 1' 1' 5'	30 cycles
NADH					
L4178a H4980	5'-CARCTWATACACYTACTATGAAA-3' 5'-ATTTTTCGTAGTTGGGTTTGRTT-3'	Macey et al. (1998)	96°C 94°C 40°C 70°C 72°C	5' 35'' 35'' 2'30'' 5'	30 cycles
CYTB					
L14724 H15175	5'-TGACTTGAARAACAYCGTTG-3' 5'-CCCTCAGAATGATATTTGTCCTCA-3'	Palumbi (1996)	96°C 94°C 55°C 72°C 72°C	5' 1' 1' 1' 5'	30 cycles
L15437 H15915	5'-CATGAAACTGGATCAAACAACCC-3' 5'-GTCTTCAGTTTTTGGTTTACAAGAC-3'	Fu (2000)	96°C 94°C 50°C 72°C 72°C	5' 1' 1' 1' 5'	30 cycles
MC1R					
MC1R-PF MC1R-PR	5'-GGCNGCCATYGTCAANAACCGGAACC-3' 5'- CTCCGRAAGGCRTAAATNATGGGGTCCAC-3'	Buades et al. (2013)	92°C 92°C 56°C 72°C 72°C	5' 30'' 30'' 1'30'' 5'	40 cycles
RAG1					
RAG-fo RAG-R1	5'- GAAAAGGGCTACATCCTGG-3' 5'-AAAATCTGCCTTCCTGTTATTG-3'	Mayer & Pavlicev (2007)	94°C 95°C 52°C 72°C 72°C	2' 10'' 15' 1' 7'	35 cycles

APOBE28					
APOBE28 F APOBE28 R	5'-TGCGGGAGGAATAYTTTGA-3' 5'-TCTATTCTRAGCTCTCCTTSRCGAA-3'	Rodriguez et al. (2017a)	95°C 95°C 49-60°C 72°C 72°C	2' 35'' 35'' 95'' 5'	35 cycles
KIAA2018					
KIAA2018 F KIAA2018 R	5'-TGCGGGAGGAATAYTTTGA-3' 5'-TCTATTCTRAGCTCTCCTTSRCGAA-3'	Rodriguez et al. (2017a)	95°C 95°C 50-58°C 72°C 72°C	2' 35'' 35'' 95'' 5'	35 cycles

3.1.3. Data analyses

3.1.3.1. Genetic variability

Basic genetic diversity parameters: number of sequences (N), variable positions (S), number of haplotypes (h), number of differences pairwise (k), haplotype diversity (Hd) and nucleotide diversity (π) were calculated with DnaSP v.6 (Librado & Rozas, 2009) for both concatenated mitochondrial and phased nuclear. Sequence divergences between all *Podarcis* populations were calculated using the distance-based method (p-distance) in MEGA7 (Kumar et al., 2016) based on mtDNA concatenated alignment.

3.1.3.2. Phylogenetic analysis and population structure

The resulting unique mtDNA haplotypes of total *Podarcis* samples were used for phylogenetic analyses, including individuals from the Balearic Islands as an outgroup: five *P. lilfordi* and three *P. pityusensis*. The best-fit nucleotide substitution models and partitioning scheme were chosen simultaneously using PartitionFinder v2.1.1 (Lanfear et al., 2016) under different model selection (BIC, AIC or AICc). The partitioning schemes were defined manually, with branch lengths of alternative partitions "linked" or "unlinked" to search for the best-fit scheme, identified with the greater Increase in Log-likelihood score (lnL) (Table 3).

Table 3. Increase in Log likelihood (lnL) values for all the combinations of model selection to choose the best substitution model and partitioning scheme using PartitionFinder (Lanfear et al., 2016).

Model	BIC	AIC	AICc
linked	-12037.69779	-11916.62304	-11917.68439
unlinked	-12618.13059	-11904.06124	-11904.06124

Phylogenetic analyses were carried out using Maximum Likelihood (ML) and Bayesian Inference (BI) methods applying the partitions with the best substitution model, i.e. with the greatest InL value. The proportion of invariable sites (I) parameter was discarded if the favoured model incorporated both the I and the Gamma site rate heterogeneity (G) parameters, as simultaneous use of these parameters can have undesirable effects (Yang, 1993).

Maximum likelihood analyses were performed using IQ-TREE version 1.6.10 (Nguyen et al., 2014) with 10^6 bootstrap replicates based on the ultrafast bootstrap approximation (UFBoot) (Minh et al., 2013; Hoang et al., 2017) for statistical support. Bayesian analyses were carried out with MrBayes 3.2.6 (Ronquist et al., 2012).

Markov Chain Monte Carlo (MCMC) chain lengths were 10^7 generations with a sampling frequency of 10^3 generations. We used two simultaneous runs of three hot and one cold chain each. Convergence was confirmed by examining the stationarity of the lnL values of the sampled trees (Figure 18) and the observation of average standard deviations of the split frequencies being <0.01. Run characteristics such as effective sample sizes (ESS) were also assessed in Tracer v1.6 (Rambaut et al., 2018). The first 25% of each run was discarded as burn-in and the phylogenetic trees were visualised and edited using Figtree v1.4.2 (Rambaut, 2014).

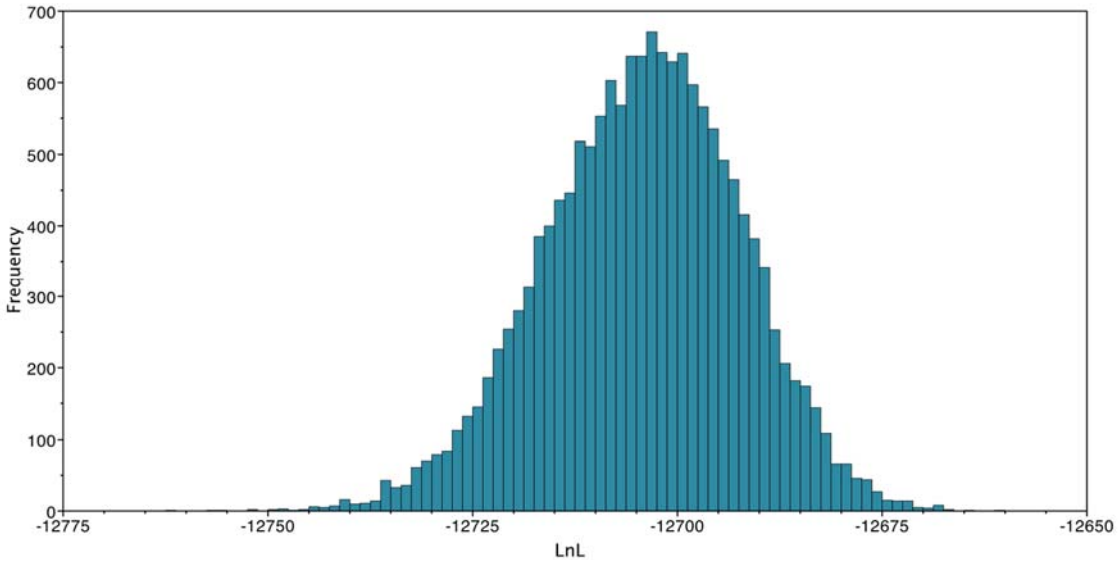


Figure 18. Increase Log-Likelihood values (lnL) of the sampled trees obtained with MrBayes.

Genealogical relationships between haplotypes of the phased nuclear genes and the complete mtDNA dataset were inferred using the TCS statistical parsimony network approach (Clement et al., 2002) with 95% connection limit implemented in the programme PopART 1.7 (http://popart.otago.ac.nz) (Leigh & Bryant, 2015).

3.1.3.3. Species tree and divergence times

In order to check what kind of clock model (strict or relaxed) is the most appropriate for divergence time dating (Brown & Yang, 2011), a standard likelihood ratio test of the molecular clock was performed for both mtDNA and nuclear datasets using MEGA7 (Kumar et al., 2016).

The species tree approach implemented in *BEAST v.2.6 (Bouckaert et al., 2019) was used to simultaneously infer phylogenetic relationships and divergence times between the different lineages of the Iberian and North African *Podarcis*. These are based on: i) all mtDNA sequences dataset, and ii) both mtDNA and phased nuclear loci (mtDNA+nuclear). The timing of separation of Balearic *Podarcis* (*Podarcis lilfordi* and *Podarcis pityusensis*) at the end of the Messinian salinity crisi (5.33 Ma) provided a time calibration (Brown et al., 2008). For i) the age of the Balearic node on the population tree was specified from a lognormal distribution with mean 1.6724 and standard deviation 0.002. Three partitions were assigned as: 1) 12S rRNA, control region, all tRNAs; 2) CYTB/ND1/ND2 1st + 2nd codon position; and 3) cytb/ND1/ND2 3rd codon position. Evolutionary models were the same as those used for MrBayes. The *BEAST MCMC sampler was run twice for $5x10^8$ generations, with one step sampled per 50,000.

A relaxed log normal clock model was specified since the molecular clock test indicated rate variation across gene trees. A coalescent Yule speciation process was used for the tree prior. For ii) the same calibration described for the mtDNA-only analyses was used. The same DNA substitution model was used for the three mtDNA partitions and the JC69 model was used for the two nuclear loci.

BEAST v.5 (Heled & Drummond, 2010) was applied as another Bayesian approach that include the estimation of divergence times among different lineages based on mtDNA dataset using also the separation of Balearic *Podarcis* as calibrator. The calibration was specified from a normal distribution (5.32, 0.01). Partitions and evolutionary models were the same as those used for MrBayes. The BEAST MCMC sampler was run twice for $5x10^8$ generations, with one step per 5,000 sampled. A relaxed log normal clock model was specified, and a Yule model was used for the tree prior.

Tracer, version 1.6 (Rambaut et al., 2018), was used in both analyses to check for convergence. Subsequent trees were combined to obtain the tree with the maximum sum of posterior clade probabilities.

3.1.3.4. Species delimitation analyses

Four species delimitation methods (three tree-based (GMYC, mPTP and bPTP) and one distance-based (ABGD)) and a Bayesian analysis of genetic structure (BAPS) were performed on the concatenated mitochondrial haplotypes dataset to determine the number of species within the Iberian *Podarcis* complex.

Generalized Mixed Yule Coalescent (GMYC) method is based on time-calibrated ultrametric phylogenetic tree and uses differences in branching rates to infer different species. The input tree was generated in BEAST v.2.4.3 (Bouckaert et al., 2014) under a relaxed clock and Yule Model tree prior. A GTR+G model of substitution was used as estimated using BIC in PartitionFinder V1.1.1 (Lanfear et al., 2016) to facilitate convergence. Analyses were run for 200 million generations, sampling every 2,000. Convergence and mixing were monitored in TRACER v.1.6 (Rambaut et al., 2014) and ESS values >200 indicated adequate sampling of the posterior. TREEANNOTATOR v2.4.3 (Bouckaert et al., 2014) was used to create a maximum clade credibility (MCC) tree using mean heights for node annotation and a 10% burn-in. The GMYC analysis was conducted using this consensus tree and the single-threshold method with the SPLITS package on R v. 3.3.2.

The multi-rate Poisson Tree Processes (mPTP) (Kapli et al., 2017) is a noncoalescent, ML, sequence-based method that models speciation in terms of number of substitutions (Zhang et al., 2013b). This method identifies changes in the tempo of branching events, where the number of substitutions between species is assumed to be significantly higher than the number of substitutions within species.

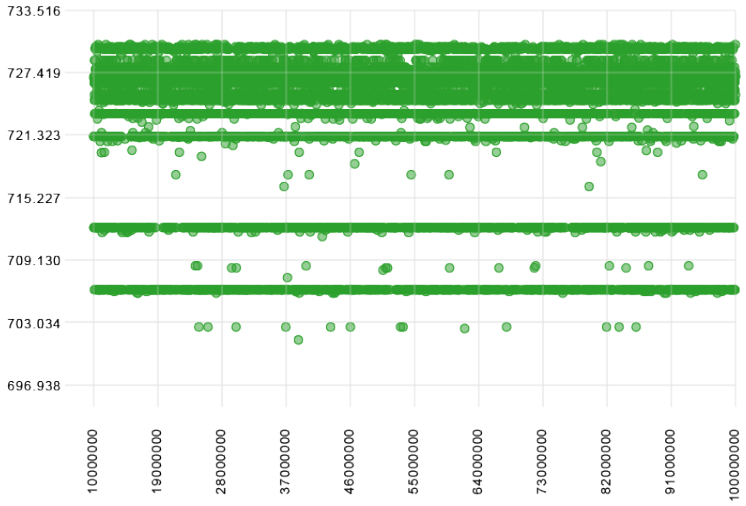


Figure 19. Plot of generation vs. log likelihood values from mPTP analyses

Analyses were performed using IQtree ML trees on the mPTP software (v.0.1.1). Confidence of the delimitation scheme was assessed using two independent MCMC runs of 10^8 steps, sampling every 10^4. Convergence was assessed by monitoring the plot of generation vs. log-likelihood (Figure 19) and the Average Standard Deviation of Delimitation Support Values among runs (0), which indicate convergence on the same distribution as it approaches to zero. MCMC analyses utilized the -multi option to incorporate differences in rates of coalescence among species.

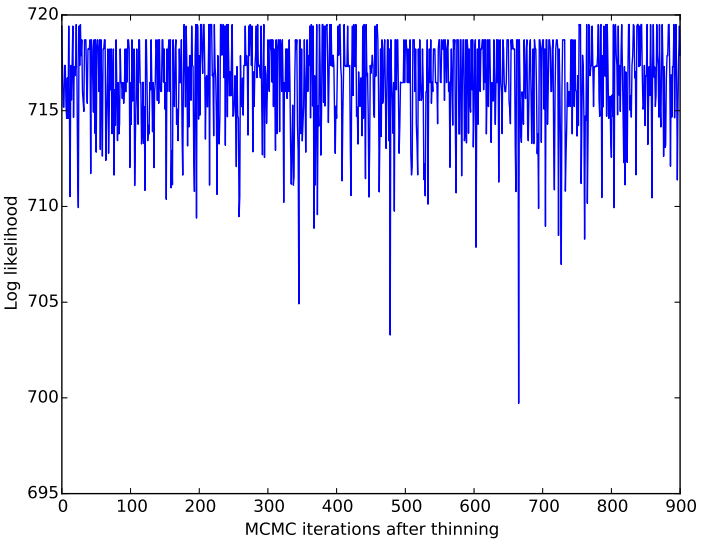


Figure 20. Plot of MCMC iteration vs. log likelihood values from bPTP analyses

Bayesian Poisson Tree Processes (bPTP) (Zhang et al., 2013b) analyses were performed using the online server (https://species.h-its.org/) and the ML tree from IQ-TREE. We ran 500,000 generations with a thinning of 500 and a burn-in of 0.1, and then assessed convergence visually using the automatically generated MCMC iteration vs. log-likelihood plot (Figure 20).

Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012) method was also run. ABGD is a computationally efficient distance-based method of species delimitation that has been shown to perform well when compared to tree-based coalescent methods (Puillandre et al., 2012; Kekkonen & Hebert, 2014; Kapli et al., 2017) and other threshold techniques (Ratnasingham & Hebert, 2013). The method seeks to quantify the location of the barcode gap that separates intra- from interspecific distances. The ABGD method was performed on the webserver (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) using the mtDNA sequence alignment as input; default values were used for prior intraspecific divergences (Pmin 0.001, Pmax 0.1, steps 10), relative gap width (1.5), and distance distribution (20). Results were compared using both JC69 and K80 models.

Genetic structure within *Podarcis* lineages was analysed using the Bayesian method in BAPS v.6 (Corander & Marttinen, 2006, Corander et al., 2013). The BAPS algorithm attempts to estimate the partition of individual sequences into clusters by analysing allele frequencies parameters for each subpopulation. This allows for the number of underlying subpopulations (K) to be estimated as part of the model fitting procedure. Genetic mixture analyses were performed with the maximal number of groups (K) set to 2-15 with three replicates. The hierBAPS (Tonkin-Hill et al., 2018) algorithm extends this approach by enabling the investigation of a population at multiple resolutions. This is achieved by initially clustering the entire dataset using the BAPS algorithm before iteratively applying the algorithm to each of the resulting clusters. BAPS attempts to find the partition of the data that maximises the posterior probability of an allocation over all other possible allocations (Tonkin-Hill et al., 2018). HierBAPS was carried out with concatenated mtDNA fragment.

Bayesian Phylogenetic and Phylogeography (BPP v.4.0) (Yang & Rannala, 2010) was used to evaluate speciation using both phased nuclear loci only and a combined dataset (complete mtDNA dataset and phased nuclear loci).

Balearic *Podarcis* was also included in the analysis. BPP is a robust means of inferring species from recently diverged lineages using multilocus data (Rannala, 2015; Leaché et al., 2018) under the multispecies coalescent model (MSC) (Rannala & Yang, 2003). This Bayesian method assumes no gene flow between species, no recombination within a locus, free recombination between loci, and neutral clock-like evolution. The program currently uses the JC69 mutation model (Jukes & Cantor, 1969) to correct for multiple hits, and mutation rate is assumed to be constant over time. The use of the JC model and the clock assumption mean that the programme should be limited to closely related species with sequence divergence not much higher than 10%. The MSC model considers genealogical heterogeneity across genome, that is, different regions of the genome have different gene tree topologies and branch length. The model also assumes that the sequences are random samples from different species. If some sequences from the same species are identical, they should all be used, and it is incorrect to use haplotypes only, which will lead to biased parameter estimates. Similarly, it is incorrect to filter loci based on bootstrap support values and use only those loci with high phylogenetic signal (Yang, 2015; Flouri et al., 2018).

BPP implements a reversible jump Markov Chain Monte Carlo (rjMCMC) search to estimate the sum of posterior probability (PP) of all species delimitation models considered. Speciation models' PP can be affected by the prior distributions chosen for the ancestral population size (θ) and root age (τ). A range of different prior scenarios were used (Table 4), including small population size (θ) and deep divergence (τ), or large population size and shallow divergence that favours more conservative models containing fewer species (Yang & Rannala, 2010). The programme can be used to conduct different analyses, specified using two variables in the control file.

Table 4. Different priors used in BPP analysis.

Priors	
θ	**τ0**
G (3. 0.02)	G (3. 0.004)
G (3. 0.02)	G (3. 0.2)
G (3. 0.02)	G (3.0.02)
G (3. 0.2)	G (3.0.02)
G (3. 0.2)	G (3. 0.2)
G (3. 0.002)	G (3. 0.004)
G (3. 0.002)	G (3. 0.04)

1) A00 (speciesdelimitation = 0, speciestree = 0): estimation of the parameters of species divergence times and population sizes under the MSC model when the species phylogeny is given (Rannala & Yang, 2003);

2) A01 (speciesdelimitation = 0, speciestree = 1): inference of the species tree when the assignments are given by the user (Rannala & Yang, 2017);

3) A10 (speciesdelimitation = 1, speciestree = 0): species delimitation using a user- specified guide tree (Yang & Rannala, 2010; Rannala & Yang, 2013);

4) A11 (speciesdelimitation = 1, speciestree = 1): joint species delimitation and species tree inference of unguided species delimitation (Yang & Rannala, 2014; Rannala & Yang, 2017).

We used the A11 model, which jointly infers the species tree and species delimitation (Rannala & Yang, 2017). The assignment of individuals to hypothesised species was based on the mtDNA lineages identified. The MCMC chain was run for 10^4 steps (following a burn-in of 2,000 steps), sampled every 25 steps. Each analysis was run three times to confirm that they converged on the same posterior.

Finally, a Bayesian multispecies coalescent method implemented in BEAST v2.5 (Bouckaert et al., 2019), called STACEY v1.8.0 (Jones, 2017), was carried out to inference a species delimitation and a species phylogeny.

This method provides a complementary analysis that enables the species delimitation results obtained with BPP to be ensured. STACEY provided better convergence of the MCMC chain than the similar approach within *BEAST (Heled & Drummond, 2010). Unlike BPP, this approach implements a Birth-Death-Collapse model, which eliminates the need for a rjMCMC algorithm, and uses different population size parameters which are integrated out, so it is not necessary to define them previously (as a prior).

STACEY was performed with a combined dataset with the three mtDNA loci and the two phased nuclear loci. The JC69 substitution model and a strict clock were specified for each. Two independent runs of 450 million generations of the MCMC chains were performed sampling every 5,000 steps. Convergence was checked in Tracer 1.7 (Rambaut et al., 2018) and posterior trees from both runs were combined (the first 20% of trees from each run were removed as burn-in). The resulting species trees were processed in SpeciesDelimitationAnalyser (Jones, 2017) with a collapse height of 0.0001 (the same value used in STACEY analysis) and default similarity cut-off (0.9). Posterior trees were combined to obtain a maximum sum of clade credibilities.

3.2. Gymnesian Islands' endemic lizard: *Podarcis lilfordi*

3.2.1. Samples

The Balearic Islands endemic lizard *P. lilfordi* has been studied based on two methodological approaches. On the one hand, we obtained the genetic structure and performed a phylogenetic analysis of all known subspecies that inhabit Mallorca, Menorca and Cabrera islands by means of mitochondrial and nuclear genes. On the other hand, a genome-wide analysis was performed using ddRADseq on *P. lilfordi* populations with different morphological and ecological characteristics, like skin coloration or population size.

Figure 21. Locations of all *P. lilfordi* from Balearic Islands used in these studies. Locations indicated in red were also used in the genome-wide analysis.

To accomplish the first objective, 117 samples of *P. lilfordi* from previous works were studied (25 from Mallorca, 47 from Menorca and 45 from Cabrera); moreover, sequences from previous studies of nine specimens of *P. pityusensis* from Ibiza were included as an outgroup. Twenty-five defined subspecies of *P. lilfordi* are represented in this sampling (Figure 21 and Table 5). The population from the Bay of Palma (Mallorca), at Porrassa Islet, is considered an introduced population. The genome-wide analysis was carried out with 94 samples from 10 different locations across Mallorca, Menorca, and Cabrera islands (Figure 21). Populations were chosen based on their colouration (melanic and non-melanic) and the number of individuals in these populations (ranging between tens and hundreds of thousands). Data concerning population size of all chosen populations was obtained from updated information (Pérez-Mellado et al., 2008, Pérez-Mellado, 2009). The phylogeographic situation of the chosen populations was taken into consideration, ensuring that they belonged to the same clade so as to minimise the effect of geographic isolation (Brown et al., 2008; Terrasa et al., 2009a, 2009b; Rodríguez et al., 2013). Between 4 and 12 individuals were captured from each locality and tail-tips were stored in DNAgard® Tissue (Biomatrica) (Figure 22) or ethanol. The Regional Ministry for the Environment (Conselleria de Medi Ambient), Government of the Balearic Islands, approved both captures and field protocols.

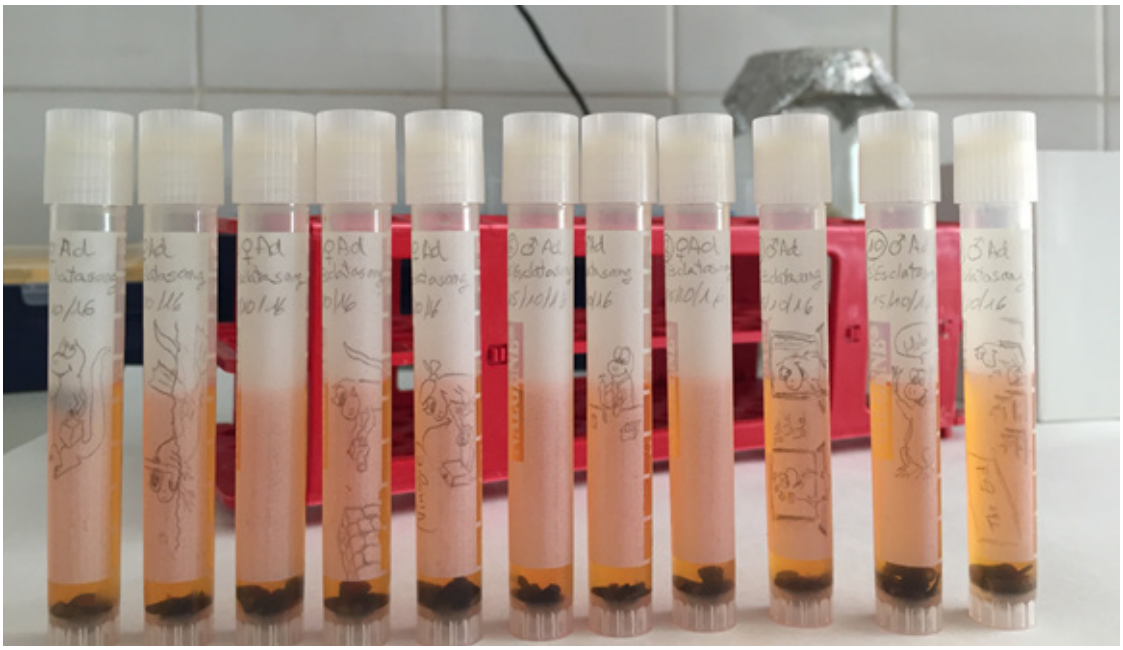


Figure 22. Collection tubes with tail tissue stored in DNAgard Tissue to ensure a high quality genomic DNA extraction. Artist: Ana Pérez-Cembranos.

Table 5. Location, population, subspecies, number of samples and GenBank Accession numbers of *P. lilfordi* samples used in the study based on mitochondrial and nuclear genes. Populations marked with an asterisk (*) were also used in genome-wide study.

Location	Population	Supspecies	N	12S	RC	CYTB/1	CYTB/2	NADH	MC1R
Mallorca	Colomer*	*P. l. colomi* (Salvador, 1979)	4	EF694760	EF694775/76	EF990524/25	EF694801/02	EU006737-39	JX126659-63
	Dragonera*	*P. l. gigliolii* (Bedriaga, 1879)	4	EF694761	EF694773	EF990519-21	EF694799	EU006730-33	JX126624/28-32
	Malgrat Petit	*P. l. hartmanni* (Wetsttein, 1937)	2	EF694762	EF694774	EF990522	EF694799	EU006734	JX126637
	Malgrat Gran	*P. l. hartmanni* (Wetsttein, 1937)	2	EF694762	EF694774	EF990522	EF694799	EU006734	JX126625/36
	Toro	*P. l. toronis* (Hartmann, 1953)	3	EF694761	EF694774	EF990523	EF694800	EU006735	JX126633-35
	Caragol	*P. l. jordansi* (Müller, 1927)	2	EF694760	EF694771	EF990517	KF003362	EU006728	MT044521
	Guardia	*P. l. jordansi* (Müller, 1927)	2	EF694760	EF694771	EF990517	KF003362	EU006728	JX126627/64/65
	Moltona	*P. l. jordansi* (Müller, 1927)	2	EF694760	EF694771/72	EF990517/18, EF694798	KF003362	EU006728/29	JX126626/66/67
	Pelada	*P. l. jordansi* (Müller, 1927)	2	EF694760	EF694771	EF990517	KF003362	EU006728	
	Porrassa	Introduced	2	EF694760	EF694772	EF990518	EF694798	EU006736	
Menorca	Aire*	*P. l. lilfordi* (Günther, 1874)	2	EF694766	EF694787	EF990546	EF694810	EU006756	JX126638/39
	Addaia Gran	*P. l. addayae* (Eisentraut, 1928)	5	EF694766	EF694786/87	EF990543/44	EF694809	EU006756	
	Addaia Petita	*P. l. addayae* (Eisentraut, 1928)	3	EF694766	EF694787/88	EF990543/45	EF694809	EU006756	JX126647
	Colom*	*P. l. brauni* (Müller, 1927)	3	EF694766	EF694787/88	EF990543/49	EF694809	EU006756	
	Bledes Menorca	*P. l. sargantanae* (Eisentraut, 1928)	2	EF694766	EF694787	EF990543	EF694812	EU006756	
	Codrell Gran	*P. l. codrellensis* Pérez-Mellado & Salvador 1988	2	EF694766	EF694788	EF990543	EF694809	EU006756	JX126641/42
	Codrell Petit	*P. l. codrellensis* Pérez-Mellado & Salvador 1988	3	EF694766	EF694788	EF990543	EF694809	EU006756	JX126643-45
	Carbó	*P. l. carbonerae* Pérez-Mellado & Salvador, 1988	3	EF694766	EF694788/89	EF990543/47/48	EF694809/11	EU006756	
	Mel	Undescribed	4	EF694766	EF694786/88	EF990543	EF694809/13	EU006761/63	
	Porros*	*P. l. porrosicola* Pérez-Mellado & Salvador, 1988	2	EF694766	EF694788	EF990543	EF694809	EU006758	
	Rovells	*P. l. sargantanae* (Eisentraut, 1928)	3	EF694766	EF694787/88	EF990543	EF694809	EU006756/58	
	Sargantana	*P. l. sargantanae* (Eisentraut, 1928)	3	EF694766	EF694787/92	EF990543	EF694809	EU006761-63	
	Tusqueta	*P. l. sargantanae* (Eisentraut, 1928)	5	EF694766	EF694788	EF990543	EF694809	EU006758/63	
	Sanitja	*P. l. fenni* (Eisentraut, 1928)	4	EF694766	EF694790/91	EF990543	EF694809	EU006760/61	
	Aguiles	Undescribed	1	EF694766	EF694787	EF990543	EF694809	EU006757/63	
	Rei*	*P. l. balearica* (Bedriaga, 1879)	2	EF694766	EF694787	EF990550/51	EF694810	EU006756/59	JX126646
Cabrera	Cabrera Far*	*P. l. kuligae* (Müller, 1927)	2	EF694764	EF694782	EF990531/36	AM747719	EU006743/45	JX126623/48-51
	Cabrera Port*	*P. l. kuligae* (Müller, 1927)	4	EF694760/64	EF694772/80/81	EF990531-34	AM747719, EF694806, KF003362	EU006736, EU006743/44	JX126652-54
	Miranda	*P. l. kuligae* (Müller, 1927)	3	EF694760/64	EF694772/82/83	EF990532/37	KF003362	EU006746-48	
	Foradada*	*P. l. fahrae* (Müller, 1927)	3	EF694760/63	EF694777	EF990526	EF694803	EU006740	JX126655-58
	Pobre	*P. l. pobrae* (Salvador, 1979)	2	EF694760	EF694772	EF990527	KF003362	EU006736	
	Plana	*P. l. planae* (Müller, 1927)	3	EF694760	EF694772/78	EF990526	EF694798/804, KF003362	EU006729/36, EU006741	
	Esponja	*P. l. espongicola* (Salvador, 1979)	3	EF694760	EF694779	EF990528	EF694804	EU006741	
	Redona	*P. l. conejerae* (Müller, 1927)	2	EF694760	EF694772	EF990526/29	KF003362	EU006742	
	Conillera	*P. l. conejerae* (Müller, 1927)	1	EF694760	EF694772	EF990526	KF003362	EU006736	
	Rates	*P. l. kuligae* (Müller, 1927)	2	EF694764	EF694782	EF990538	AM747719	EU006743	
	Ses Bledes	*P. l. nigerrima* (Salvador, 1979)	4	EF694764	EF694782	EF990531	AM747719	EU006743/49	

	Fonoll	*P. l. kuligae* (Müller, 1927)	4	EF694764	EF694782/84	EF990532	AM747719	EU006743/50
	Imperial	*P. l. imperialensis* (Salvador, 1979)	3	EF694764	EF694782	EF990531	AM747719	EU006743/51/52
	Estell des Coll	*P. l. estelicola* (Salvador, 1979)	2	EF694764	EF694782	EF990531	AM747719	EU006743/55
	Estell de Fora	*P. l. estelicola* (Salvador, 1979)	2	EF694765	EF694785	EF990536	EF694807	EU006753
	Xapat	*P. l. xapaticola* (Salvador, 1979)	2	EF694765	EF694785	EF990536/39	EF694807	EU006753
	Esclatasang*	*P. l. xapaticola* (Salvador, 1979)	3	EF694764/65	EF694785	EF990541/42	EF694807/08	EU006753/54
	Outgroup							
Ibiza (outgroup)	Espardell	*P. pityusensis*		EF694769	EF694794	JX852083	JX852082	JX852121
	Espalmador			EF694768	EF694794	JX852083	JX852048	JX852121
	Caragoler			EF694768	EF694794	JX852060	JX852092	JX852124
	Alga			EF694768	EF694794	JX852083	JX852075	JX852119
	Negra Nord			EF694768	EF694794	JX852072	JX852092	JX852132
	Eivissa			EF694768	EF694794	JX852056	JX852056	JX852121
	Es Vedrà			EF694768	JX852107			
	Formentera (SFX)			EF694768	EF694794	JX852052	JX852082	JX852129
	Formentera (PT)			EF694768	EF694794	JX852083	JX852082	JX852121

3.2.2. Mitochondrial and nuclear genes

GenBank sequences of the four non-overlapping mtDNA (12S, RC, CYTB and NADH) genes for the total number of individuals (117) and for the nine *P. pityusensis* were used. Moreover, 45 GenBank sequences of MC1R gene were included (Table 5). Nuclear data were phased using the PHASE algorithm (Stephens et al., 2001) with DnaSP v.6 (Rozas et al., 2017).

3.2.2.1. Phylogenetic analyses and population structure

To establish the level of divergence between *P. lilfordi* subspecies, a distance-based analysis (p-distance) was performed based on mtDNA dataset using MEGA7 (Kumar et al., 2016). Two different datasets were used for phylogenetic analyses: 1) concatenated four mitochondrial DNA fragments providing an alignment of 2,382 bp length; and 2) phased nuclear alignment (MC1R) with a length of 720 bp.

The same methodology described previously in section 3.1.2 was applied for mtDNA dataset alignment and substitution model and partitioning schemes selection, which consisted of one partition: [HKY+G+I]. Only the Bayesian analysis was performed with MrBayes 3.2.1 (Ronquist et al., 2012). Two runs of 10^6 steps with a sampling frequency every 10^3 generations were performed. Sufficient number of generations was confirmed by examining the stationarity of the log likelihood (lnL) values of the trees sampled and a value of average standard deviations of split frequencies lower than 0.01. Results were analysed in Tracer v1.7 (Rambaut et al., 2018) to assess convergence and effective sample sizes (ESS) for all parameters. A burn-in of 25% was applied and phylogenetic trees were visualized and edited using Figtree v1.4.2 (Rambaut, 2014). Genealogical relationships between the different MC1R haplotypes were inferred by performing a TCS statistical parsimony network (Clement et al., 2002) with a 95% limit connection using the programme PopART 1.7 (http://popart.otago.ac.nz) (Leigh & Bryant, 2015).

3.2.3. Genome wide analyses (ddRADseq)

3.2.3.1. DNA extraction and quality control

Extraction of high-quality genomic DNA was conducted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's standard protocol (Purification of Total DNA from Animal Tissues) with modifications. Briefly, between 22 and 59 mg of tail tissue was cut up into small pieces and disrupted with the TISSUE MASTER 125 homogenizer (OMNI International, GA, USA) in 180 µl of ATL buffer. Twenty microliters of Proteinase K (20mg/µl) was added; tubes were mixed thoroughly by vortexing and incubated at 56°C until complete lysis. During incubation, samples were vortexed occasionally. Next, a specific step of copurification with RNase A Solution/ RNase A, DNase and protease-free (Promega) was performed, 15 µl of RNAse A (4 mg/ml) was added and samples were incubated at room temperature for 5 min. Then, the same volume (200 µl) of AL buffer and 100% ethanol was added and the mixture was vortexed thoroughly and pipetted into a column placed in a collection tube. Centrifugation with 7,155 g for 1 min was carried out using 2-16 K Centrifuge (Sigma, Germany), and flow-through and collection tube were discarded. The column was placed in a new tube collection and 500 µl of AW1 buffer was added followed by centrifugation in the same conditions. Again, the collection tube and flow-through were discarded. The previous step was repeated using AW2 buffer this time and centrifugation with 20,000 g for 3 min. Finally, an elution step was performed by placing the column in a new tube and adding 200 µl of buffer AE onto the membrane.

After 1 min incubation at room temperature followed by a centrifugation step with 7,155 g for 1 min, genomic DNA was stored at -20°C. Quality of the genomic DNA samples was evaluated by running 2 µl of each sample on 2% agarose gel electrophoresis against a 1kb ladder (Figure 23) and determining the A260:A280 ratio using Nanovue Plus Spectrophotometer (GE Healthcare). Genomic DNA was quantified with Thermo Fisher Scientific Qubit 3.0 Fluorometer using Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific) (Table 6).

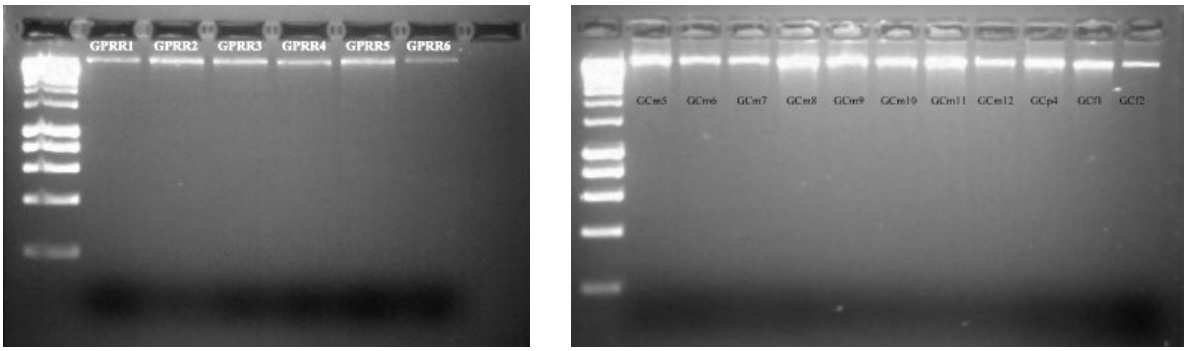


Figure 23. Agarose gels (2%) showing genomic DNA samples against a 1 kb ladder to assess the quality of the samples.

Table 6. Information about the samples used in the genome-wide analysis: Sex, state, location, initial amount of tissue used in the extraction, concetration and quality ratio values. Abbreviations: ad, adult; subad, subadult and juv, juvenile.

Sample	Sex	State	Location	Tissue (g)	Nanovue (ng/µl)	ratio A260/280	Qubit (ng/µl)
GPRR1	macho	ad	Porros (Fornells)	0,0252	20,5	1,830	9,42
GPRR2	macho	ad	Porros (Fornells)	0,0251	31,5	1,853	22
GPRR3	macho	ad	Porros (Fornells)	0,0269	19,5	1,884	8,52
GPRR4	macho	ad	Porros (Fornells)	0,0255	24	1,811	15
GPRR5	macho	ad	Porros (Fornells)	0,0279	20	1,681	18,4
GPRR6	hembra	ad	Porros (Fornells)	0,0270	17	1,838	10,9
GPRR7	hembra	ad	Porros (Fornells)	0,0236	26,5	1,779	13,8
GPRR8	hembra	ad	Porros (Fornells)	0,0290	26	1,739	18,3
GPRR9	hembra	ad	Porros (Fornells)	0,0269	21,5	1,700	13,7
GPRR10	macho	ad	Porros (Fornells)	0,0245	12,7	1,438	8,82
GRE1	macho	ad	Rei (Menorca)	0,0224	20	1,633	18,9
GRE2	macho	ad	Rei (Menorca)	0,0263	15,9	1,719	8
GRE3	macho	ad	Rei (Menorca)	0,0394	21	1,772	11,6
GRE4	hembra	ad	Rei (Menorca)	0,0380	28,5	1,727	17,7
GRE5	hembra	ad	Rei (Menorca)	0,0456	35	1,750	13,8
GRE6	hembra	ad	Rei (Menorca)	0,0487	21,5	1,792	15,9
GRE7	hembra	ad	Rei (Menorca)	0,0275	24,5	1,707	12,7
GRE8	hembra	ad	Rei (Menorca)	0,0276	22	1,660	10
GRE9	hembra	subad	Rei (Menorca)	0,0259	16	1,561	7,84
GRE10	macho	ad	Rei (Menorca)	0,0300	14,3	1,723	8,78
GA1	hembra	ad	Aire (Menorca)	0,0277	8,9	1,648	5,54
GA2	hembra	ad	Aire (Menorca)	0,0297	17,5	1,750	6,6
GA3a	macho	ad	Aire (Menorca)	0,0287	27	1,698	24,4
GA3b	macho	ad	Aire (Menorca)	0,0295	26	1,694	15,8
GA4a	macho	ad	Aire (Menorca)	0,0252	14	1,722	9,64
GA4b	macho	ad	Aire (Menorca)	0,0256	18	1,809	15,7
GA5	macho	subad	Aire (Menorca)	0,0270	12,1	1,729	10,8
GA6	macho	ad	Aire (Menorca)	0,0270	15,2	1,783	12
GA7	hembra	subad	Aire (Menorca)	0,0280	24,5	1,782	22
GA8	macho	ad	Aire (Menorca)	0,0286	20,5	1,647	13,8
GA9	hembra	subad	Aire (Menorca)	0,0300	33	1,784	28,4
GA10	hembra	subad	Aire (Menorca)	0,0247	14,8	1,609	7,68

GCm1	hembra	ad	Colom (Menorca)	0,0245	21,5	1,552	4,86
GCm4	macho	ad	Colom (Menorca)	0,0289	20	1,709	11,3
GCm5	hembra	subad	Colom (Menorca)	0,0305	25,5	1,706	24,8
GCm6	hembra	subad	Colom (Menorca)	0,0250	17,5	1,691	15,3
GCm7	hembra	subad	Colom (Menorca)	0,0280	20,5	1,723	14,3
GCm8	hembra	subad	Colom (Menorca)	0,0290	31,5	1,703	24
GCm9	hembra	subad	Colom (Menorca)	0,0298	25	1,639	26
GCm10	hembra	subad	Colom (Menorca)	0,0323	20	1,826	19,6
GCm11	hembra	ad	Colom (Menorca)	0,0406	24,5	1,738	65,2
GCm12	macho	ad	Colom (Menorca)	0,0280	23	1,704	25,2
GCp4	macho	ad	Cabrera (Port)	0,0252	14,1	2,029	17,8
GCp5	hembra	subad	Cabrera (Port)	0,0313	24,5	2,042	34,2
GCp6	hembra	ad	Cabrera (Port)	0,0323	23	1,991	35,2
GCp7	macho	ad	Cabrera (Port)	0,0263	24	1,868	23
GCp8	hembra	ad	Cabrera (Port)	0,0311	23,5	1,918	25,2
GCp9	macho	ad	Cabrera (Port)	0,0353	42,5	1,848	53,4
GCp10	hembra	subad	Cabrera (Port)	0,0318	18,5	1,659	11
GCp11	hembra	ad	Cabrera (Port)	0,0345	16	1,693	30,2
GCp12	macho	ad	Cabrera (Port)	0,0366	16,6	1,632	12,9
GCp13	hembra	ad	Cabrera (Port)	0,0312	22,5	1,751	20,2
GCp14	hembra	subad	Cabrera (Port)	0,0302	28,5	1,770	24,2
GCp15	hembra	ad	Cabrera (Port)	0,0268	17	1,667	9,12
GCf1	macho	ad	Far d'ensiola (Cabrera)	0,0296	19,5	1,718	11,9
GCf2	hembra	ad	Far d'ensiola (Cabrera)	0,0286	17,5	1,606	12,4
GCf3	macho	ad	Far d'ensiola (Cabrera)	0,0275	24	1,839	16,8
GCf4	macho	ad	Far d'ensiola (Cabrera)	0,0335	24	1,739	13,4
GCf5	hembra	subad	Far d'ensiola (Cabrera)	0,0311	17	1,667	13,6
GCf6	macho	subad	Far d'ensiola (Cabrera)	0,0289	22,5	1,718	18
GCf7	macho	ad	Far d'ensiola (Cabrera)	0,0387	15,6	1,681	9,7
GCf8	hembra	ad	Far d'ensiola (Cabrera)	0,0262	24	1,714	10,3
GCf9	macho	ad	Far d'ensiola (Cabrera)	0,0367	24,5	1,696	17,3
GCf10	macho	subad	Far d'ensiola (Cabrera)	0,0351	26	1,694	16,3
GFo1	hembra	ad	Na Foradada (Cabrera)	0,0263	16,2	1,929	15,1
GFo2	macho	ad	Na Foradada (Cabrera)	0,0316	33	1,886	25,6
GFo3	macho	ad	Na Foradada (Cabrera)	0,0288	27,5	1,897	16,9
GFo4	macho	ad	Na Foradada (Cabrera)	0,0358	38,5	1,833	25,4
GFo5	hembra	ad	Na Foradada (Cabrera)	0,0272	37	1,850	26
GFo6	macho	ad	Na Foradada (Cabrera)	0,0332	34,5	1,865	29
GFo7	macho	ad	Na Foradada (Cabrera)	0,0283	16,2	1,785	26
GFo8	macho	ad	Na Foradada (Cabrera)	0,0282	17	1,581	30,4
GFo9	macho	ad	Na Foradada (Cabrera)	0,0318	22,5	1,613	23,4
GFo10	hembra	ad	Na Foradada (Cabrera)	0,0393	25	1,718	21,6
GEt1	hembra	ad	S'Esclatasang (Cabrera)	0,0378	12,1	1,674	33,6
GEt2	hembra	ad	S'Esclatasang (Cabrera)	0,0267	16,6	1,657	10,7
GEt3	hembra	ad	S'Esclatasang (Cabrera)	0,0388	23,5	1,754	21,2
GEt4	hembra	ad	S'Esclatasang (Cabrera)	0,0372	32,5	1,711	13,9
GEt5	hembra	ad	S'Esclatasang (Cabrera)	0,0300	26	1,962	21,2
GEt6	macho	ad	S'Esclatasang (Cabrera)	0,0317	19,5	1,822	25,2
GEt7	macho	ad	S'Esclatasang (Cabrera)	0,0287	19,5	1,840	49
GEt8	hembra	ad	S'Esclatasang (Cabrera)	0,0255	18	1,674	20,4
GEt9	macho	ad	S'Esclatasang (Cabrera)	0,0330	18,5	2,000	20,2
GEt10	macho	ad	S'Esclatasang (Cabrera)	0,0374	20,5	1,767	18,9
GEt11	macho	ad	S'Esclatasang (Cabrera)	0,0303	13,4	1,841	28,8
GD1	hembra	juv	Casa Guarda (Dragonera)	0,0356	37	1,805	31,4
GD2	macho	ad	Cap Llebeig (Dragonera)	0,0316	82,5	1,833	76,4
GD3	macho	ad	Tramuntana (Dragonera)	0,0334	94,5	1,853	102
GD4	macho	ad	Na Pòpia (Dragonera)	0,0337	60,5	1,779	52,2
GC1	hembra	ad	Es Colomer	0,0324	81	1,800	106
GC2	hembra	ad	Es Colomer	0,0322	102,5	1,847	85,4
GC3	macho	ad	Es Colomer	0,0326	72,5	1,790	104
GC4	macho	ad	Es Colomer (cima)	0,0262	85	1,809	101
GPRR10r	macho	ad	Porros (Fornells)	0,0590	23	1,776	13,5

3.2.3.2. ddRAD-seq library construction

The ddRADseq library preparation requires the normalisation of DNA concentration (equivalent concentrations) between all the samples analysed. To normalise the samples to a concentration of 20 ng/µl, all samples with concentrations below this value were concentrated in SpeedVac Vacuum Concentrator (Thermo Fisher Scientific). This tool is a combination between centrifugation and a vacuum chamber, enabling samples to be dried. The volume required to obtain a concentration of 20 ng/µl was marked on the tube which was centrifuged in the vacuum concentrator for 40-45 min at 35°C. Excess liquid was removed, and normalised concentrations were obtained (Figure 24).

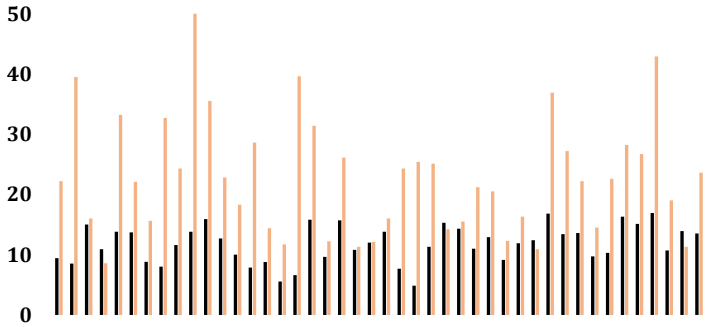


Figure 24. Comparison between initial (black) and final (orange) concentration after performed the speed vacuum method.

DNA was sent to Floragenex (Eugene, Oregon, USA) for preparation of the RAD tag library and performance of Illumina paired-end sequencing. Briefly, 100-500 ng of total genomic DNA was digested using 5 units *PstI* (CTGCAG) and 5 units *MseI* (TTAA) for at least 1 hour at 37°C. Following digestion, the mixture was heated at 85°C for 10 minutes. Sample-specific *PstI* X adapters contained a unique 5-nt sample identification tag (barcode) adjacent to the *PstI* restriction site overhang for the identification of individual samples, and were designed such that each sample identification tag differed by at least two bases from all other tags (Table 7). Floragenex added a control sample (*Saccharomyces*). Adapter ligation was then performed using a universal *PstI* adapter (top: 5'-GATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTxxxxTGCA-3'-; bottom: 5'-yyyyAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATC-3'-) where xxxx and yyyy are the barcode and its reverse complement, respectively and universal P7 *MseI* adapter (top: 5'-CAAGCAGAAGACGGCATACGAG-3'-; bottom: 5'-TACTCGTATGCCGTCTTCTGCTTG-3'-NH2) (Truong et al., 2012) for 3 hours at 37°C. After adapter ligation, a PCR was performed with the objective of amplifying targets with a 5' *PstI* site and a 3' *MseI* site. A 3' primer that selects for fragments which have a "TC" in front of the *MseI* site was used to decrease complexity. The library was paired-end sequenced (101 pb) on an Illumina HighSeq 4000 in the University of Oregon Genomics and Cell Characterization Core Facility.

Quality of raw reads was checked by FASTQC v.0.11.5, which provides some analysis modules (Figure 25). The first module shows some basic statistics regarding the data: total number of sequences, sequences labelled as poor quality, sequence length, and GC percentage. The second module is a plot where the position in read (bp) is represented against quality scores (Phred), reporting sequence quality per base. A similar module shows the per sequence quality score to discover whether a subset of sequences has low quality values. Per base sequence content indicates the proportion of each base position (G, A, T, C). The per sequence GC content module, measures GC content across the length of each sequence and compares it to a modelled normal distribution of GC content. The number of uncalled bases (N) is measured in the per base N content module by plotting the percentage of Ns at each position in the sequence (<5%).

Table 7. List of identifications barcodes for all the samples used in the genome-wide analyses.

Barcode	Sample Identifier	Barcode	Sample Identifier	Barcode	Sample Identifier
AACACATGCAG	GPRR1	AAGGCATGCAG	GCm1	ACCGCATGCAG	GFo1
ACTATATGCAG	GPRR2	AGGACATGCAG	GCm4	ATACGATGCAG	GFo2
CACTGATGCAG	GPRR3	CAGTAATGCAG	GCm5	CCAGTATGCAG	GFo3
CGCAGATGCAG	GPRR4	CGGAAATGCAG	GCm6	CGTGAATGCAG	GFo4
CTTACATGCAG	GPRR5	GAAGTATGCAG	GCm7	GCGCGATGCAG	GFo5
GGTACATGCAG	GPRR6	GTACTATGCAG	GCm8	GTCGCATGCAG	GFo6
GTTATATGCAG	GPRR7	TCAAGATGCAG	GCm9	TACTTATGCAG	GFo7
TCTAAATGCAG	GPRR8	TCCACATGCAG	GCm10	TGAACATGCAG	GFo8
TTGCGATGCAG	GPRR9	ACACTATGCAG	GCm11	ACCTTATGCAG	GFo9
ACTCCATGCAG	GPRR10	AGGCGATGCAG	GCm12	ATAGTATGCAG	GFo10
CAGACATGCAG	GRE1	CATAGATGCAG	GCp4	CCATAATGCAG	GEt1
CGCCAATGCAG	GRE2	CGGTCATGCAG	GCp5	CGTTGATGCAG	GEt2
CCTCAATGCAG	GRE3	GAATAATGCAG	GCp6	GCGGTATGCAG	GEt3
CTCGGATGCAG	GRE4	GTAGGATGCAG	GCp7	GTCTTATGCAG	GEt4
GTTCCATGCAG	GRE5	TACAGATGCAG	GCp8	TAGAAATGCAG	GEt5
TATGTATGCAG	GRE6	TCCGTATGCAG	GCp9	TGACGATGCAG	GEt6
AACGTATGCAG	GRE7	ACAGGATGCAG	GCp10	ACGAAATGCAG	GEt7
ACTGAATGCAG	GRE8	AGGTAATGCAG	GCp11	ATATAATGCAG	GEt8
CAGCGATGCAG	GRE9	CCAACATGCAG	GCp12	AATGGATGCAG	GEt9
CGCGCATGCAG	GRE10	CGTATATGCAG	GCp13	CTATGATGCAG	GEt10
CTTGTATGCAG	GA1	GCCATATGCAG	GCp14	GCGTAATGCAG	GEt11
GGTGTATGCAG	GA2	GTCAGATGCAG	GCp15	GTGAAATGCAG	GD1
GTTGAATGCAG	GA3a	TACCAATGCAG	GCf1	TAGTCATGCAG	GD2
TGCCTATGCAG	GA3b	TCCTAATGCAG	GCf2	TGAGTATGCAG	GD3
AACTAATGCAG	GA4a	ACCAGATGCAG	GCf3	ACGTCATGCAG	GD4
ACTTGATGCAG	GA4b	ATAACATGCAG	GCf4	ATTGCATGCAG	GC1
CAGGTATGCAG	GA5	CCACGATGCAG	GCf5	CAACAATGCAG	GC2
CGCTTATGCAG	GA6	CGTCCATGCAG	GCf6	CTGAGATGCAG	GC3
GAACGATGCAG	GA7	GCGACATGCAG	GCf7	GGAATATGCAG	GC4
GCTCTATGCAG	GA8	GTCCAATGCAG	GCf8	GTGTCATGCAG	GPRR10r
GGCTGATGCAG	GA9	TACGCATGCAG	GCf9	TGATAATGCAG	FGXCONTROL
TCAGAATGCAG	GA10	TCGCAATGCAG	GCf10		

Sequence length distribution is also displayed in FASTQC and uniformity in sequence length (101 bp) is indicated. Sequence duplication levels were also calculated. A low level of duplication indicates a high level of coverage of the target sequence, but a high level is more likely to indicate an enrichment bias as PCR over amplification. Overrepresented sequences and overrepresented K-mers are the two last modules analysed by FASTQC. The first one indicates whether a single sequence is very overrepresented in the set, which may indicate a highly biologically significant region, low diversity, or contamination (2n reads 0.1-1%).

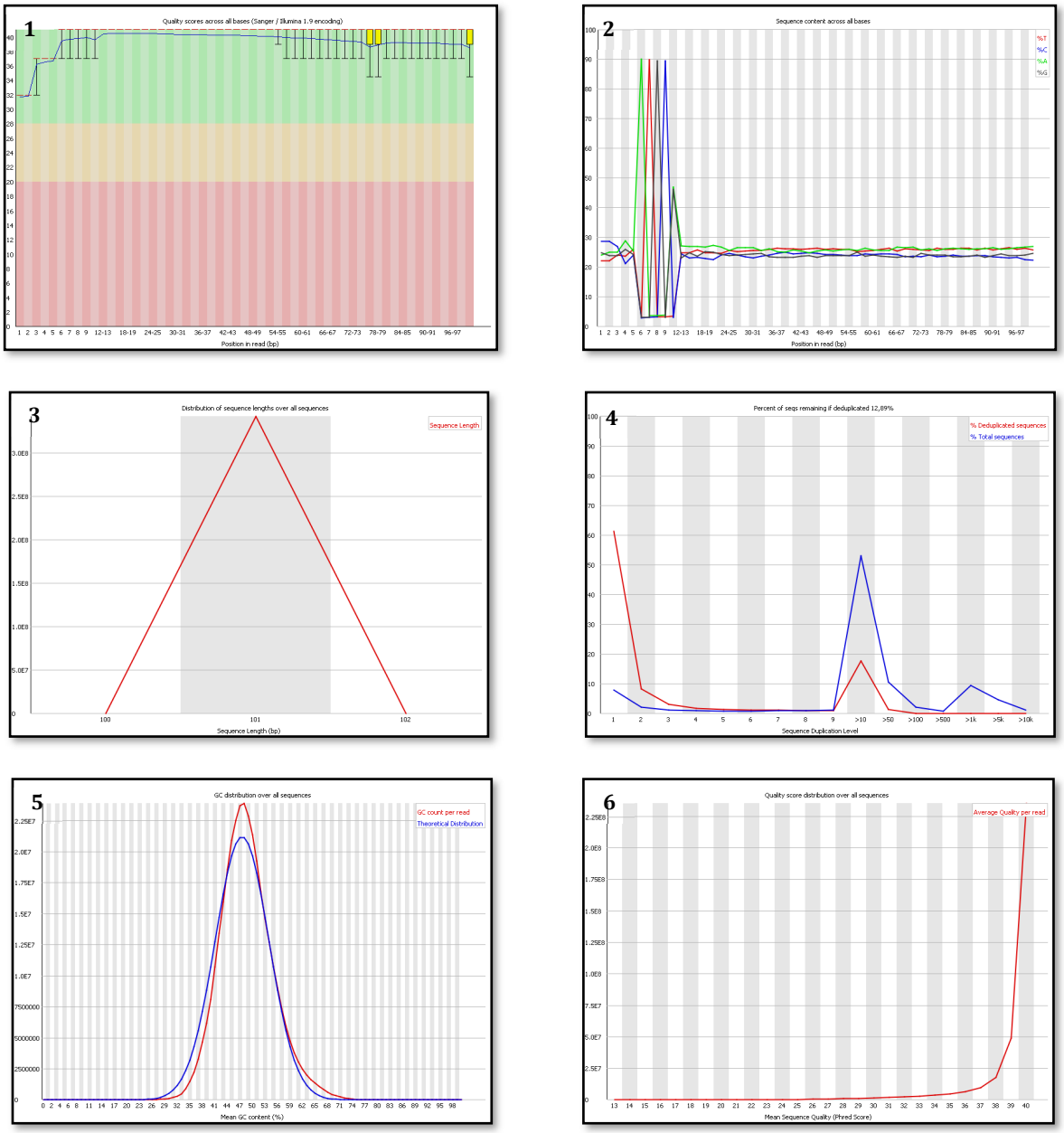


Figure 25. FASTQC resulting plots: per base: 1) sequence quality, 2) sequence content, 3) sequence length distribution, 4) sequence duplication levels; per sequence: 5) GC content and 6) quality score.

3.2.3.3. ddRAD-seq data analysis and SNP calling

After checking the quality of our library, analyses were performed using STACKS v2.4 (Catchen et al., 2013) and *denovo_map.pl* pipeline to identify SNPs across all sequences and summarise them in a single VCF file. First, individual samples were cleaned and demultiplexed with pipeline *process_radtags*. This programme examines the reads and checks the presence of barcodes and the restriction enzyme cut site (RAD cutsite), in order to subsequently demultiplex the data. Demultiplexing consists of separating the reads according to the different barcodes to get clusters of reads with the same barcode (same individual).

Process_radtags also has a filtering step that removes any read with an uncalled base (-c), discards reads with low quality scores (phred score lower than 10) (-q) (Ewing & Green, 1998), and allows mismatches in barcodes (--barcode_dist_2). In general, higher stringency is needed for *de novo* assemblies as compared to alignments to a reference genome. The *process_radtags* command used was as follow:

```
>process_radtags -1 /Volumes/My
Passport/ddRADseq_25_4_2018/C794_1.fastq.gz -2 /Volumes/My
Passport/ddRADseq_25_4_2018/C794_2.fastq.gz -b
barcodes_names.txt -o /Volumes/My
Passport/process_radtags_disable/ --renz_1 pstI --renz_2 mseI -c
-q -r --barcode_dist_2 nucleotide --disable_rad_check
```

After cleaning and demultiplexing data, another filtering step was performed using VCFtools 0.1.17 (Danecek et al., 2011) to detect individuals with a high percentage of missing data (Figure 26). Individuals with more than 70% missing data were ruled out. Clean reads were used to perform a *de novo* RAD assembly using the *denovo_map.pl* pipeline in STACKS v2.4 (Catchen et al., 2013). This pipeline was chosen due to the unavailability of a reference genome. In the *de novo* pipeline, reads were subjected to different analysis processes: the *ustacks* programme assembles short-read sequences into matching stacks within each individual. The minimum number of raw reads required to form an initial stack is defined by the -m parameter, the maximum distance (in nucleotides) allowed for merging stacks into putative loci is defined by -M parameter, the maximum distance allowed to align secondary reads to primary stacks is defined by -N. In this study, -N parameter was set as default: M+2. The *cstacks* creates a catalogue, using the set of samples processed by the *ustacks,* merging all consensus loci across individuals. The number of mismatches allowed between loci to build the catalogue is defined by the -n parameter.

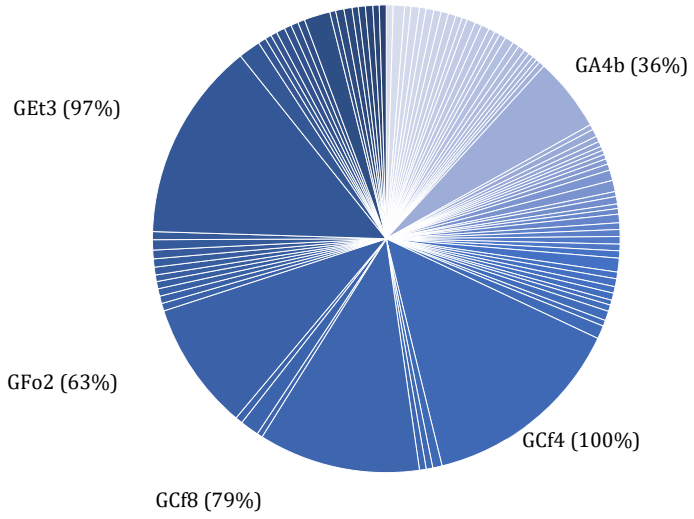


Figure 26. Plot representing percentage of missing data for all the individuals genotyped. Identification and percentage are indicated for individuals with the highest values.

Table 8. Parameters chosen for the *de novo* pipeline in Stacks (Catchen et al., 2013).

Program	Parameters	Description	Parameter trials
ustacks Assembled identical raw sequence reads into Stacks	-m	The minimum number of raw reads required to form an initial stack	3
	-N	The maximum nucleotide distance allowed between stacks and secondary reads	Default (M+2)
	-M	The maximum nucleotide distance allowed between stacks	1-6
cstacks Creates a catalogue of consensus loci by compiling stacks identified across individuals.	-n	The number of mismatches allowed between loci to build the catalogue	1-6

The optimal values for the different parameters in the *denovo_map.pl* must be chosen with an appropriate methodology, because they frequently have a significant effect on the building and quality of the resulting loci. Values for the -M and -n parameters, were identified following the r80 optimization approach (Paris et al., 2017) with a subset of one individual per population (10). The *de novo* pipeline was run six times with different combinations of these parameters (M1n1, M2n2, M3n3, M4n4, M5n5, M6n6). Paris et al. (2017) found that the approach when setting the -M and -n parameters to the same value obtained the best results. Setting -m parameter as 3 (Table 8) seems to be an optimal value for other studies based on different biological systems (Paris et al., 2017; Rochette & Catchen, 2017). The r80 optimization method includes a filtering step using the population program in Stacks, consisting of retaining only loci shared among a minimum of 80% of samples across all populations. The number of polymorphic loci (Figure 27) shared among at least 80% of samples across all populations were compared between runs. After optimal parameters were identified (M=n=3), the *de novo* pipeline was run again with optimal settings.

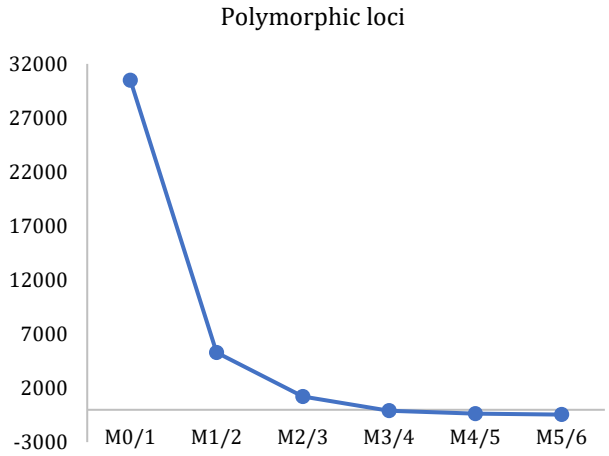


Figure 27. Plot of the number of new polymorphic loci (R80) obtained for different parameter trials in stacks.

The final output was filtered using *population* setting the -R parameter as 0.80, the minimum number of populations a locus must be present in so as to process this locus (-p parameter) as 10 and with a minimum allele frequency (MAF) of 0.05. Only the first single SNP per RAD tag was called using *–write-single-snp* to reduce linkage disequilibrium effects. The SNP dataset was exported to Bayescan (Foll & Gaggiotti, 2008) format using PGDSpider version v 2.1.1.5 (Lischer & Excoffier, 2012) and to Admixture v1.3.0, (Alexander et al., 2009) format using PLINK v1.90 (Chang et al., 2015).

3.2.3.4. Data analyses

3.2.3.4.1. Genetic diversity

Population specific genetic parameters were calculated for the dataset with first-single SNPs. Using STACKS v4.2 (Catchen et al., 2013), number of private alleles (PA), nucleotide diversity (π), observed heterozygosity (Ho), expected heterozygosity (He), and inbreeding coefficient (F_{IS}) were obtained for all positions and only for variant position. Moreover, the F_{ST} parameter that estimates genome-wide divergence between populations was calculated with a p-value correction ($p<0.001$) to exclude non-statically significant F_{ST} measures. Hierfstat R package (Goudet & Jombart, 2015) was used as another approach to calculate some genetic diversity parameters and corroborated the patterns of variability among these populations. Ho and He were obtained using the *basic.stats* function and population specific F_{IS} and F_{ST} were determined using the *boot.ppfis* and *boot.ppfst* functions respectively with a bootstrap confidence interval of 1,000 replicates. Pairwise F_{ST} following Weir & Cockerham (1984) was calculated using the *genet.dist* function and represented on heatmaps created with ggplot2 in R (Wickham et al., 2016) for both datasets, single SNPs and only outliers SNPs.

3.2.3.4.2. Population structure

The genetic relationship and population structure across all individuals were analysed following three approaches with different assumptions. These analyses were performed based on all the variability represented by the first-single SNPs dataset and the variability given by SNPs under selection (outliers).

Discriminant Analysis of Principal Components (DAPC) is a method to obtain a global representation of divergence between populations and consequently know their structure. This method finds genetic clusters in populations and is executed using the R package *adegenet* (Jombart & Ahmed, 2011). Optimal number of clusters (k) was determined by the lowest Bayesian Information Criterion (BIC) and a successive K-means algorithm to compare different numbers of groups (k=1 to k=10). In the first step, a Principal Components Analysis (PCA) was applied to examine the differentiation among individuals irrespective of population of origin. All biallelic genome sites coded as 0, 1, or 2, corresponding to homozygosity of the reference allele, heterozygosity, or homozygosity of the alternative allele, respectively. In the second step, individuals were grouped by population for the Discriminant Function Analysis (DFA), which maximises among-group relative to within-group variation. The optimal number of principal components (PCs) retained for the DAPC analysis was assessed determining the highest Mean Successful Assignment Rate and the lowest Root Mean Square Error (RMSE) using the cross-validation (CV) procedure in *adegenet* with a 10% hold-out set and 100 replicates (Table 9 and Figure 28).

Admixture v1.3.0 (Alexander et al., 2009) was used to estimate population structure assuming different hypothetical numbers of clusters (k). The programme includes a CV procedure to identify the value of K associated with the best predictive accuracy model. CV was set to 10-fold to compare different number of K (ranging from 2 to 10) in which lower CV error value indicates the most likely number of clusters.

Neighbor-Joining (NJ) trees were inferred using MEGA7 (Kumar et al., 2016) based on pairwise F_{ST} distances obtained by both approaches.

Table 9 . Number of PCs used in DAPC analysis resulting from the cross-validation method. Optimal values are highlighted in bold.

PCs	Mean Successful Assignment		Root Mean Squared Error	
	All SNPs	Outlier SNPs	All SNPs	Outlier SNPs
5	0.8683	0.7963	0.1460	0.2136
10	0.9890	0.8913	0.0234	0.1168
15	**0.9950**	0.9188	**0.0158**	0.0926
20	0.9897	0.9327	0.0246	0.0803
25	0.9888	**0.9333**	0.0242	**0.0799**
30	0.9817	0.9310	0.0351	0.0826
35	0.9732	0.9213	0.0432	0.0894
40	0.9753	0.9083	0.0389	0.1052
45	0.9657	0.9178	0.0525	0.1032
50	0.9138	0.8803	0.1024	0.1336
55	0.8397	0.8547	0.1755	0.1620
60	0.6330	0.6768	0.3928	0.3553
65	0.6365	0.7620	0.3766	0.2563

3.2.3.4.3. Adaptive divergence: Test of selection

The single SNPs dataset was analysed to find signals of divergent selection among the ten *P. lilfordi* populations, by means of two different approaches: (i) F_{ST} method to detect candidate loci under selection using BayeScan (Foll & Gaggiotti, 2008); and (ii) association analysis to test the correlation between genetic variation and environmental variables through a Redundancy Analysis (RDA) using *vegan* R package (Oksanen et al., 2018).

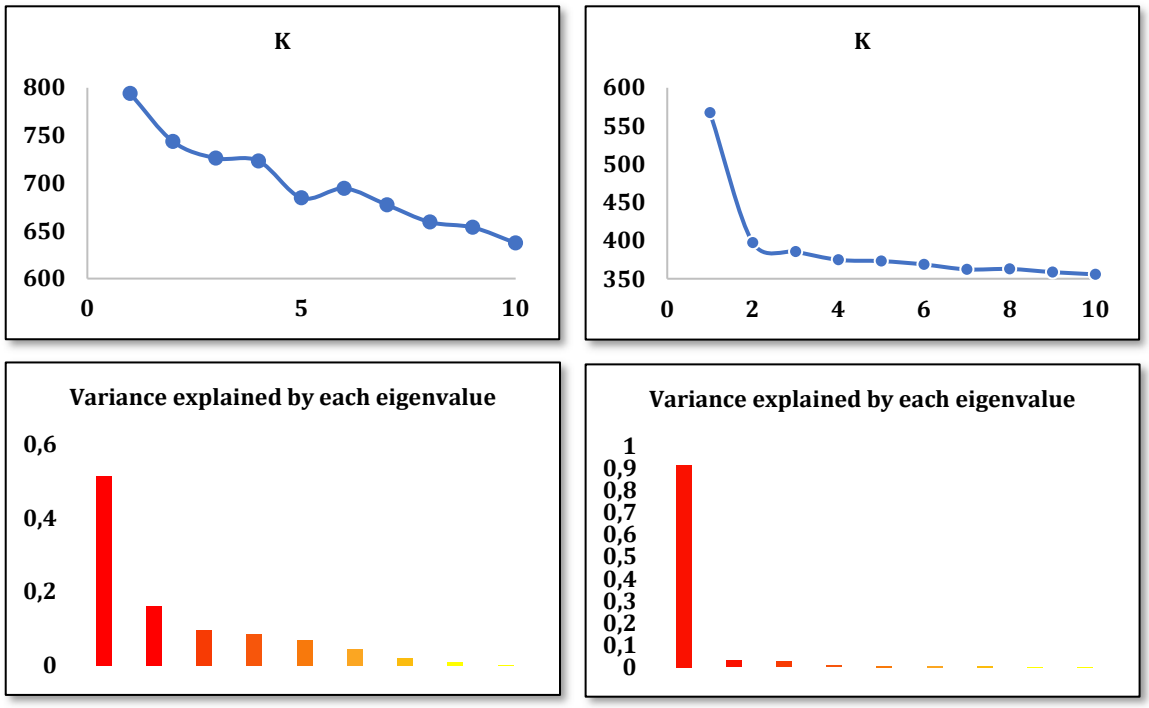


Figure 28. Graphics with optimal k number (above) and proportion of variance explained by each eigenvalue (below) for all SNPs (left) and only outliers (right).

It has been suggested that BayeScan (Foll & Gaggiotti, 2008) is more efficient in recognition of outlier loci with low false positive rate (Pérez-Figueroa et al., 2010). The method implements a rjMCMC approach that can move between a selection model, containing a population-specific component and a locus-specific component, and a model with just a population-specific component (no selection). The posterior probability for selection at a locus is determined by the proportion of MCMC samples that include the model with the locus-specific component. Twenty pilot runs of 5,000 steps were used in the MCMC approach. A burn-in of 50,000 iterations was used, followed by 5,000 iterations using a sampling interval of 10. The prior odds specified for the ratio of neutral: selected sites were set at 100, reflecting that the model with selection might be around 100 times less likely than the model without selection (Lotterhos & Whitlock, 2014). This prior can have considerable influence on the number of sites detected, so runs were also carried out with 1:10 and 1:1000 proportions. Outliers were identified from the results using the R code supplied with BayeScan and significance was determined under a false discovery rate of 5% to avoid overestimating the percentage of outliers.

3.2.3.4.4. Correlation with environmental variables (RDA)

Redundancy analysis (RDA) (Forester et al., 2018) using *vegan* R package (Oksanen et al., 2018) was performed based on the entire genome variability, represented by the single SNPs dataset and using the subset of outlier identified with BayeScan. This analysis was carried out to estimate the proportion of genetic variance that is explained by the history of populations (mtDNA genetic distances), genetic drift (effective population size), and/or divergence selection (ecology variables) (Table 10). Genetic distances based on mtDNA (Terrasa et al., 2009a) alignment (2,382 bp) for all the populations studied were calculated with MEGA7 (Kumar et al., 2016) using Tamura-Nei model with a bootstrap of 1,000. Resulting distances were transformed into vectors using the function *pcoa* in the *ape* package in R with Lingoes correction for negative eigenvalues (Legendre & Anderson, 1999).

In RDA analysis, missing genotypes were imputed on the SNP loci dataset by replacing them with the most common genotype across all individuals. The global significance of the model and the percentage of genetic variance explained by each variable separately were tested with an ANOVA using 1,000 permutations.

Multicollinearity occurs when two or more predictors in a model are correlated providing redundant information. Multicollinearity was measured by variance inflation factor (VIF), which measures the proportion by which the variance of regression coefficient is inflated in the presence of other explanatory variables. VIFs above 20 indicate strong collinearity and above 10 should be examined and avoided if possible. Therefore, variables with VIF > 10 were ruled out (Liu et al., 2017; Borcard et al., 2018).

Table 10. Population name, subspecies designation, presence or absence of melanism, number of samples used in this study (N); mtDNA clade to which they belong according to Terrasa et al. (2009a), geographical localization (latitude and longitude), population density (individual per hectarea (id/ha)) and population size about all populations used in RDA analysis.

Population	Subspecies	Melanism	N	mtDNA clade	Latitude	Longitude	Density (id/ha)	Estimated population size
Aire	*P. l. lilfordi*	melanic	12	1	39.802	4.290	3,100	77,500
Colom	*P. l. brauni*	Non-melanic	10	1	39.961	4.278	1,255	58,107
Porros	*P. l. porrosicola*	Non-melanic	10	1	40.038	4.138	1,189	54
Rei	*P. l. balearica*	Non-melanic	10	1	39.886	4.287	500	1,845
Cabrera (harbour)	*P. l. kuligae*	Non-melanic	12	2	39.151	2.933	507	534,888
Cabrera (Lighthouse)	*P. l. kuligae*	melanic	10	2	39.130	2.921	507	5,171
Esclatasang	*P. l. xapaticola*	melanic	11	2	39.125	2.942	1,786	714
Foradada	*P. l. fahrae*	melanic	10	3	39.206	2.978	1,179	1,356
Dragonera	*P. l. gigliolii*	Non-melanic	4	4	39.583	2.319	531	132,875
Colomer	*P. l. colomi*	melanic	4	3	39.583	3.131	4,007	10,017

3.3. MC1R gene expression analyses

3.3.1. Samples

Two types of *P. lilfordi* samples were used in this analysis, tail tissue and eggs at different incubation times. The fieldwork on these protected species was authorised by the Regional Ministry of Agriculture, Environment and Territory (Conselleria d'Agricultura, Medi Ambient i Territori), Government of the Balearic Islands, who specifically authorised capture, release of lizards, and removal of a tail tip for DNA/RNA analysis.

Tail samples were caught by careful noosing following the same method described in section 3.1.1. After sample collection, tissues were immediately stored in RNAlater® Tissue Protect Tubes (Qiagen) to preserve RNA integrity and subsequently stored at -20°C when they arrived at the laboratory. Thirty-three tail samples of *P. lilfordi* populations with different skin colouration (Table 11) were obtained. Ten of them, five individuals from Foradada and five from Esclatasang islands, both located in the Cabrera archipelago, presented a melanic phenotype characterised by a dark blue abdomen and black dorsal region. The remaining 23 individuals belonged to populations with non-melanic pigmentation and included 11 individuals from Dragonera and 12 from Cabrera harbour (Figure 29). In the Dragonera population, we found individuals with an orange abdomen and brown dorsal while individuals from Cabrera harbour presented a green or blue colouration in the dorsal region and a bright blue abdomen (Figure 30).

Table 11. Number of analysed samples (N), phenotype and location.

Tissue type	Population	Location	N	Phenotype
Tail tip	Dragonera	Mallorca	11	Non-melanic
	Cabrera harbour	Cabrera	12	Non-melanic
	Foradada	Cabrera	5	Melanic
	Esclatasang	Cabrera	5	Melanic

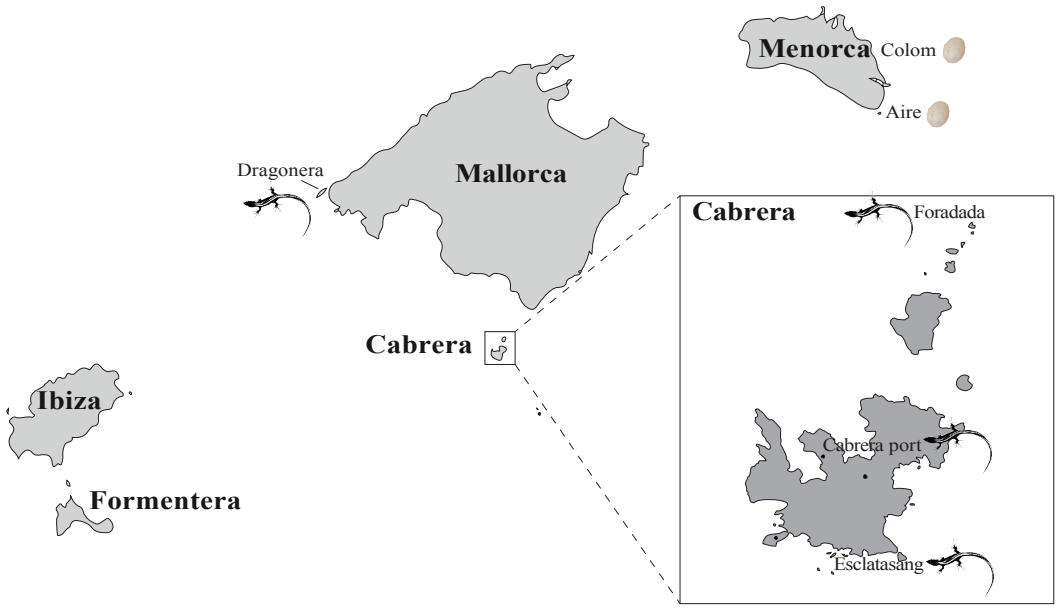


Figure 29. Location of *P. lilfordi* studied populations. Populations where tail samples were used are indicated with a lizard figure, while populations where eggs samples were recollected are indicated with an egg figure.

Figure 30. Different skin coloration in *P. lilfordi* studied populations. Dragonera (1), Cabrera harbour (2), Esclatasang (3) and Foradada (4). Source: Marta Bassitta and Valentín Pérez-Mellado.

Figure 31. Individuals from Colom island (left) and from Aire island (right) in Menorca. Source: Valentín Pérez-Mellado and Ana Pérez-Cembranos.

To obtain egg samples, six gravid females from a melanic (Aire) and six from a non-melanic (Colom) population, both situated in Menorca island (Figure 29 and 31), were caught in the same breeding season (September-January) in 2018. Gravid females were selected by the presence of copulation marks. They were kept in individual terraria with a sand substrate, and provided with water and food ad libitum. Four of the six females from Aire and three of the six females from Colom laid eggs 8-14 days after their capture. All the females were released 15 days (Aire) and 22 days (Colom) after capture. We obtained 11 eggs from Aire and 8 eggs from Colom. We checked for laying females twice a day. Most times, the eggs were on top of the sand, but sometimes the female covered the eggs with damp sand. All the eggs were carefully extracted, keeping the upper side of each egg in that position. A small point was painted on their upper face (Figure 32) with a non-toxic marker. Eggs were always maintained with this point side up, avoiding their rotation. Eggs from each individual female were marked with a different colour. Eggs were then buried 1 cm in a mix 1:4 of deionized water and vermiculite, with no contact between them and with no contact with the walls of the container. Eggs were kept in an incubator at 27˚C and 70% relative humidity. Development of embryos was stopped at three different stages by putting them in a vial with RNAlater (Qiagen) and then stored at -20˚C. An initial stage was considered for eggs stored on the same day of laying (time 0). A middle stage of development was represented by eggs stopped at 8 to 16 days after laying, and a final third stage of development was considered between 17 to 28 days of development (Table 12).

Figure 32. Eggs with the mark on their upper face to ensure the incubation position.

Table 12. Number of eggs and different incubation time for the non-melanic (Colom) and the melanic population (Aire).

Incubation (days)	0	8	11	14	16	17	19	25	28
Colom (eggs)	2	2	2	2					
Aire (eggs)	3				2	1	1	1	3

3.3.2. RNA extraction

Special precautions were taken into account when extracting RNA due to the known instability that this nucleic acid presents. A special area was designated for working only with RNA and working surfaces were cleaned with 100% ethanol. Electrophoresis tanks were cleaned with 1% SDS, rinsed with milli-Q H2O, then rinsed with 100% ethanol and immersed in 3% H2O2 for 10 minutes. Finally, the tank was rinsed with DEPC-treated H2O (Ishikawa, 1977). Before starting the extraction, all the material was autoclaved and treated with UV light to ensure decontamination. All the centrifugation steps were performed at 22°C and 5,000 g using 2-16 K Centrifuge (Sigma). Total RNA was extracted from tail tissue and eggs (Figure 33) using the RNeasy® Midi Kit (Qiagen). Samples were completely thawed before use and between 0.15 and 0.25 g of tissue were used.

Tissue was cut into small pieces and 4 ml of RTL buffer were added (10 µl of ß-mercaptoethanol for each millilitre of RTL buffer) in 15 ml tubes. All the mixture was homogenised using Ultra-turrak T8 (IKA Labortechnik) by performing five rounds of 15 seconds with 15-second breaks between each one. Then, 65 µl of Proteinase K (20 mg/ml) was added and mixed by pipetting and tubes were incubated at 55°C for 20 minutes. After incubation, samples were centrifuged for ten minutes. After centrifugation, 3 ml of supernatant was recovered, thereby avoiding the thin layer that forms on top of the supernatant, and pipetted into a new 15 ml tube. Posteriorly, 0.5 volumes of 100% ethanol were added and mixed well by pipetting. A maximum of 4 ml of the sample was pipetted into an RNeasy midi column (supplied in the kit) placed into a 15 ml tube, and centrifuged for five minutes. Flow-through was discarded.

Figure 33. *Podarcis* eggs from Aire Island with 0 days (above) and 28 days (below) of incubation.

An extra purification step is recommended in RNA applications that are sensitive to small amounts of DNA (i.e. RT-PCR). Hence, a DNase digestion was performed using RNase free-DNA set (Qiagen) as follows. A volume of 2 ml of RW1 buffer was added and a five-minute centrifugation was carried out. After discard flow-through, a mixture with 20 µl of RNase-free DNase and 140 µl of RDD buffer was pipetted into the column and incubated at room temperature for 15 minutes. Then 2 ml of RW2 buffer were added, incubated at room temperature for 5 minutes and a 5 minutes centrifugation step was performed. The flow-through was discarded and 2.5 ml of buffer RPE were added into the column and centrifugate for 2 minutes to wash the column. A second washing step with 2.5 ml RPE was repeated but for 5 minutes this time. To elute, the RNeasy column was transferred into a new collection tube (15 ml) and 200 µl of RNase free water was added directly onto the membrane. It was left for one minute at room temperature and then centrifuged for three minutes. The flow-through was kept since it contained the high-quality RNA.

Concentration and 260/280 quality ratio of the extracted RNA was estimated using Nanovue Plus (GE Healthcare). Sample quality was also assessed with 1.5% agarose gel, stained with ethidium bromide. The general method for loading the RNA samples into the agarose gel consists of mixing a certain concentration of RNA with a 1:5 proportion of DNA loading buffer 5X (Bioline), heating in AccuBlock Digital Dry Bath (LabNet, USA) at 70°C and subsequently chilling on ice. To determine the optimal method to visualized RNA bands in agarose gel, different RNA samples concentrations were tested, as well as the order in which heat was supplied to the samples. The three first samples were heated and chilled before being mixed with loading buffer, while the other four were mixed first (Table 13 and Figure 34). Finally, all the samples were mixed with loading buffer prior to being heated at 70°C for 5 min and then chilled on ice for 3 min, whenever possible using RNA concentrations greater than 200 ng. Gels were visualised using UV MiniBis BioImaging System (DNR Bio-Imaging Systems) and HyperLadder 1kb (Bioline) was used as molecular weight marker. A high-quality RNA sample should show two clear bands corresponding to the most abundant RNAs (28S and 18S) in eukaryotes (Figure 34).

Table 13. Different conditions to determine the optimal method to loading RNA samples into agarose gel.

	RNA (ng)	70°C (min)	ice (min)
1	100	1	3
2	200	1	3
3	500	1	3
4	100	5	3
5	500	5	3
6	100	10	3
7	500	10	3

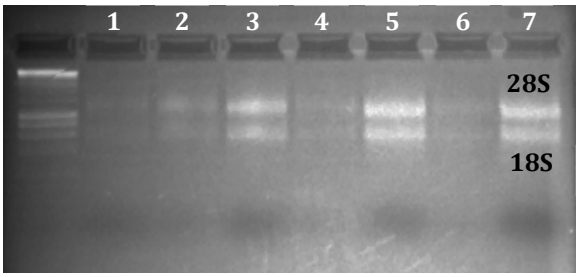


Figure 34. Agarose gel showing the resulting bands obtained under the different conditions indicated in Table 13.

Table 14. Location, presence of bands in agarose gel, concentration values and quality ratio values for egg samples used in gene expression study.

Sample	Location	Agarose gel	RNA concentration [ng/µl]	260/280 ratio
TAIL-Fo1	Foradada		-1.6	0.75
TAIL-Fo5	Foradada		0.8	6.67
TAIL-Fo6	Foradada		-3.6	1.57
TAIL-Fo7	Foradada		-1	1.32
TAIL-Fo9	Foradada	√	17.6	2.10
TAIL-Et1	Foradada	√	40	2.04
TAIL-Et6	Esclatasang		3	2.77
TAIL-Et7	Esclatasang	√	103.6	2.07
TAIL-Et8	Esclatasang	√	13.2	2.06
TAIL-Et10	Esclatasang	√	17.6	2.07
TAIL-D1r	Dragonera	√	50	2.00
TAIL-D2r	Dragonera		3.3	4.30
TAIL-D3r	Dragonera	√	18.4	2.00
TAIL-D4r	Dragonera	√	36.4	2.00
TAIL-D5r	Dragonera		7.2	2.00
TAIL-D6r	Dragonera		3.9	2.72
TAIL-D7r	Dragonera	√	18.4	2.07
TAIL-D8r	Dragonera	√	26.4	2.04
TAIL-D9r	Dragonera	√	11.2	2.02
TAIL-D10r	Dragonera	√	54.4	2.00
TAIL-D11r	Dragonera		1.6	3.00
TAIL-Cp1r	Cabrera harbour	√	25.2	2.04
TAIL-Cp2r	Cabrera harbour	√	35.2	2.05
TAIL-Cp3r	Cabrera harbour	√	26.4	2.12
TAIL-Cp4r	Cabrera harbour	√	47.2	2.03
TAIL-Cp5r	Cabrera harbour		2.2	-18
TAIL-Cp6r	Cabrera harbour		108.8	1.4
TAIL-Cp7r	Cabrera harbour	√	24.8	2.00
TAIL-Cp8r	Cabrera harbour		3.1	3.25
TAIL-Cp9r	Cabrera harbour	√	39.6	2.11
TAIL-Cp10r	Cabrera harbour	√	30.8	2.14
TAIL-Cp11r	Cabrera harbour	√	44.8	2.00

TAIL-Cp12r	Cabrera harbour	√	29.6	2.06
EGG-Colom0_1	Colom		14.4	2.11
EGG-Colom0_2	Colom		21.6	2.14
EGG-Colom8_1	Colom		4.7	2.29
EGG-Colom8_2	Colom	√	7.6	2.481
EGG-Colom11_1	Colom	√	239.6	2.08
EGG-Colom11_2	Colom	√	120.8	2.08
EGG-Colom14_1	Colom	√	336.4	2.07
EGG-Colom14_2	Colom	√	102.8	2.04
EGG-Aire0_1	Aire		29.2	2.09
EGG-Aire0_2	Aire		34	2.07
EGG-Aire0_3	Aire		59.2	2.06
EGG-Aire16_1	Aire	√	53.6	2.06
EGG-Aire16_2	Aire		112	2.06
EGG-Aire17_1	Aire	√	72.4	2.08
EGG-Aire19_3	Aire	√	133.2	2.06
EGG-Aire25_2	Aire	√	268.4	2.05
EGG-Aire28_1	Aire	√	266.8	2.05
EGG-Aire28_3	Aire	√	217.2	2.06
EGG-Aire28_4	Aire	√	172.4	2.05

The 63.6% of tail tissue and the 63.2% egg showed optimal quality values (ratio and agarose gel) indicating high integrity values of RNA and their suitability for use in posterior analyses (Table 14). All egg samples were included in the study since they showed correct concentration and quality values despite not showing a band in the agarose gel.

3.3.3. Reverse transcription

Prior to reverse transcription to obtain complementary DNA (cDNA) from extracted RNA, all samples were subjected to an extra purification step with DNase I (Sigma-Aldrich, Germany). RNA samples concentration were normalised to 100 ng to avoid experimental error (Huggett et al., 2005) to 100 ng and one sample was selected as DNA contamination control. This amount of RNA was mixed with 8 µl of RNase free-water, 1 µl of buffer 10X, and 1 µl of DNase I (1,500 Kunitz units), then all the mixture was incubated at room temperature for 15 minutes. The reaction was stopped with one microlitre of STOP elution and samples were heated at 70°C using AccuBlock Digital Dry Bath (LabNet). After that, samples were stored on ice until the next step. Reverse transcription was performed using All-in-One cDNA Synthesis SuperMix (Biotool) with the objective to select only mRNA, which is the RNA of interest for gene expression studies. All-in-One cDNA Synthesis SuperMix used a combination of oligo (dT) and random primers to reduce the bias produced near the 5' or 3' ends of cDNAs and to overcome variability that can result from using different individual primers. So, 2 µl of qRT SuperMix was added to total RNA and RNase-free water up to a volume of 10 µl. To the control sample we added 2 µl of 5x No RT Control Mix, if the control sample amplified in the qPCR could indicate DNA contamination. To obtain higher quality cDNA, RNA templates were incubated at 25°C for 10 minutes, then at 42°C for 30 minutes for extension, and finally at 85°C for 5 minutes to terminate the reaction using a SimpliAmp (Applied Biosystems) thermal cycler.

3.3.4. Gene expression analysis

Reverse transcription quantitative PCR (RT-qPCR) was carried out on the LightCycler 480 System (Roche). This method is an adequate technique to quantify gene expression for genes with low expression, degraded RNA, or samples of limited tissue (Fleige & Pfaffl, 2006). The β-actin (ACTB) gene was used as reference gene or housekeeping gene, since it is expressed constitutively in every cell, to normalise the results of MC1R gene expression. Primers used in RT-qPCR were (Fulgione et al., 2015):

For the target gene, MC1R:

Forward: 5'-TGGAGACCCTCTTCATGCTTCT-3'

Reverse: 5'-GCTGCAGATCAGCATGTCCA-3'

For the reference gene, ACTB:

Forward: 5'-GATCTGGCACCACACCTTCT-3'

Reverse: 5'-TCTTTTCTCTGTTGGCTTTGG-3'

A fragment of 101bp of the target gene MC1R was amplified. RT-qPCR was performed in a final volume of 20 µl, with 2 µl of cDNA, 0.8 µl of each primer (10 µM), 10 µl of SYBR Premix EX Taq TM II 2x (TaKaRa), and 6.4 µl of sterile water. PCR was initiated with a 30 s hold at 95 °C, followed by 45 cycles at 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 20 s. After amplification, the samples were heated to 95 °C for 5s, 65 °C for 1 m, and 97 °C, and then cooled to 40 °C for 30 s to obtain the melting curve for SYBR Green. Melting curves (Figure 35) make it possible to know whether a single PCR product has been generated or whether something else has been amplified. The fluorescent dye SYBR Green binds to the DNA double helix and exhibits fluorescence, but does not emit fluorescence when it is free or bound to simple strand DNA. For this reason, in the final step the temperature is increased, DNA strands are separated, and fluorescence decreases progressively. The melting temperature of each PCR product is characteristic and depends on GC content or PCR product length. Each sample was analysed in triplicate and no-RT and no-template controls were included in all experiments.

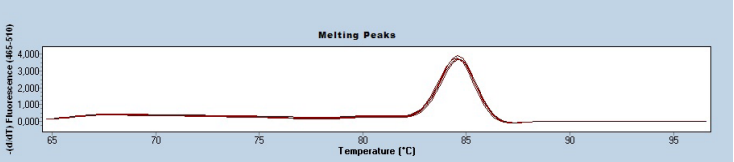


Figure 35. Melting peak indicating a single PCR product.

3.3.5. Relative quantification and statistical analysis

Relative quantification is based on determination of a target gene expression (MC1R) depending on the expression of this gene compared to a control sample or calibrator. In addition, a housekeeping gene (ACTB) is necessary as internal control and to normalise the expression of the target gene (Pfaffl, 2006). In this study, the objective was to analyse MC1R gene expression in melanic and non-melanic populations to assess whether there are different levels of expression in both adult individuals (tails) and in different development stages (eggs).

In the analysis of adult individuals based on tail samples, the calibrator sample was D10, a non-melanic form from Dragonera Island; and in the analysis involving different stages of embryonic development (eggs), the calibrator sample was from Colom Island (Colom11_1), also a non-melanic population. In

RT-qPCR a positive reaction is detected by accumulation of fluorescent signal. Ct values (cycle threshold) (Pfaffl et al., 2004) is the number of cycles at which the fluorescence emitted was higher than the fluorescence detection threshold (exceeds background level). Ct levels are inversely proportional to the amount of target gene in the sample, so lower Ct values indicate higher levels of gene expression. For each sample, the average Ct was calculated between replicates of MC1R and normalised using the average Ct value of the reference gene, ACTB. Relative quantification was conducted using two approaches:

The standard $2^{-(\Delta\Delta CT)}$ method (Livak & Schmittgen, 2001),

$$\Delta\Delta CT = (Ct_{target} - Ct_{reference})_{Time\ X} - (Ct_{target} - Ct_{reference})_{Time\ 0}$$

Average Ct values of melanic or non-melanic samples (Time X) were normalized using reference gene (ACTB) and depending on the calibrator sample (Time 0). The results were presented as fold change ($2^{-(\Delta\Delta CT)}$), i.e. as the expression ratio. Positive values of fold change indicated an upregulation of the target gene, while a negative value represents a low expression of that gene (Livak & Schmittgen, 2001). Some factors can bias the fold change of the analysis, such as the PCR efficiency or the absence of expression. The other method used was Pfaffl method (Pfaffl, 2001), where PCR efficiencies of target and reference gene were taking into account to calculated the ratio of relative expression,

$$\text{ratio} = \frac{(E_{target})^{\Delta CP_{target}\ (control-sample)}}{(E_{ref})^{\Delta CP_{ref}\ (control-sample)}}$$

PCR efficiencies (E) were calculated by performing a standard curve using six 1:10 dilution series run in triplicate for each gene (MC1R and ACTB). PCR efficiency is affected by different factors, like the specific use of PCR machine, reagents, or primers. A tail sample from a non-melanic *P. lilfordi* population from Rei island (Menorca) was used to perform the standard curve for the gene expression experiment based on tail samples. In the analyses based on egg samples, the standard curve was carried out with a sample from the melanic population of Aire (Aire25_2) in Menorca (Figure 36).

In order to determine whether the different values found in expression levels between melanic and non-melanic groups were statistically significant or were the result achieved by chance, the p-value of each group compared in the analysis was calculated with a statistical test. A p-value lower than 0.05 is considered significant, according to the gold standard in statistics (Fisher, 1925). The statistical test used can be parametric or non-parametric. Before choosing the test to use, we needed to determine whether the distribution of the Ct values for every comparison followed a "normal" distribution. The parametric test runs under the assumption that the distribution is normal while non-parametric tests do not make such assumption. When normality is not proven, using a non-parametric test (not assuming normality) reduces the risk of misinterpretation of the results.

A Kolmogorov-Smirnov or a Shapiro Wilk's test with a $p < 0.05$ was carried out to verify whether fold change values in expression levels in melanic and non-melanic groups followed a normal distribution. If the results did not follow a normal distribution, the Mann-Whitney test was performed to compare medians between melanic and non-melanic groups. One-way ANOVA and t-test were used as a parametric method when the data seemed to follow a normal distribution. All statistical test were carried out in R (R Core Team, 2018).

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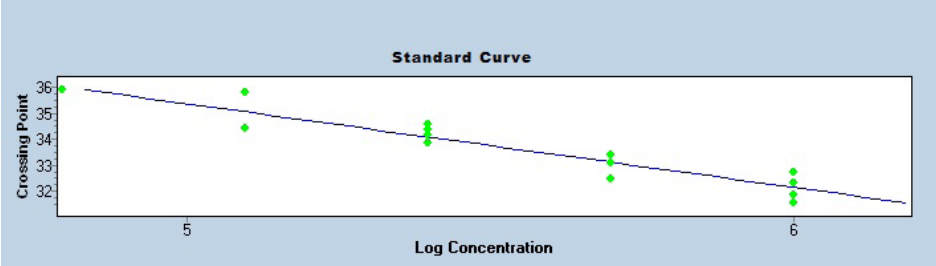

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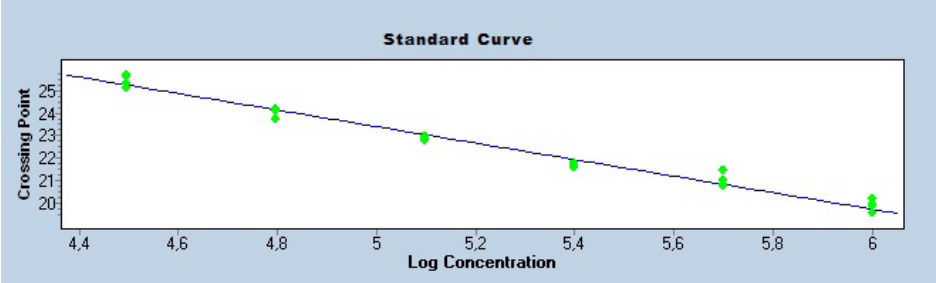

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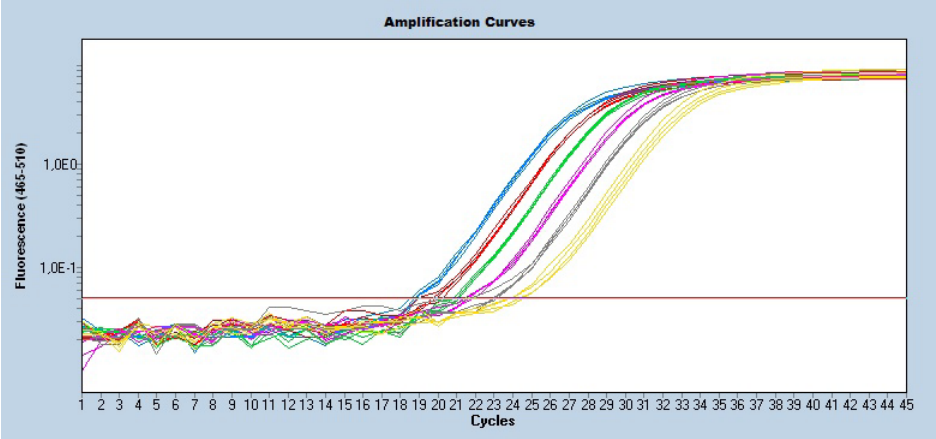

Figure 36. Standard curves for target gene, MC1R (1) and reference gene, ACTB (2) based on melanic egg sample (Aire25_2). Amplification plot (3) are created when the fluorescence signal from each sample is plotted against cycle number. That plot represents the accumulation of product over the duration of the real-time PCR experiment. The samples used in this plot are a dilution series of the reference gene (ACTB).

4

Results

CHAPTER 1

Iberian *Podarcis* Complex

Multilocus and morphological analysis of south-eastern Iberian Wall lizards (Squamata, *Podarcis*)

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Evolutionary history of wall lizards of the Columbretes archipelago

(In preparation)

Introduction

In this chapter, the *Podarcis hispanicus* complex is the object of study and to achieve the proposed goals, a total of 162 individuals from 44 different localities were used. This species complex comprises *Podarcis* populations that inhabit the Iberian Peninsula and North Africa. Seven of them have achieved species recognition: *P. muralis*, *P. carbonelli*, *P. bocagei*, *P. guadarramae* (including two subspecies: *P. g. lusitanicus* and *P. g. guadarramae*), *P. virescens*, *P. liolepis* and *P. hispanicus*. The latter represents the nominotypical form, which has been a controversial point in different studies. *Podarcis hispanicus* inhabits the SE Iberian Peninsula and three morphologically similar lineages belonging to different mitochondrial clades have been found: Valencia, Albacete/Murcia, and Galera lineages. Another interesting group within this complex is the Columbretes Islands population that was initially considered a new species (*P. atrata*) but was subsequently included in the *P. liolepis* clade.

In the first manuscript, published in the journal *Zoologica Scripta*, the results provided enough support to elevate Galera lineage to full species rank and to recognise the Albacete/Murcia lineage as the nominotypical taxon (*P. hispanicus sensu stricto*). These results were based on a large genetic dataset: four mtDNA genes (2,301 bp) and two nuclear genes (MC1R (663 bp) and RAG1 (939 bp)) with a greater number of individuals (104) and locations than previous studies.

The second study, currently in preparation, included an extensive genetic analysis based on mtDNA (2,301 bp) and four nuclear genes (the two used in the previous study plus APOBE28 (489 bp) and KIAA2018 (623 bp)) from the Columbretes Islands population. The objective was to determine the genetic variability of the *Podarcis* form that inhabits these volcanic islands and its evolutionary history.

Multilocus and morphological analysis of south-eastern Iberian Wall lizards (Squamata, *Podarcis*)

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Abstract

The phylogenetic relationships among the wall lizards of the *Podarcis hispanicus* complex that inhabit the south-east (SE) of the Iberian Peninsula and other lineages of the complex remain unclear. In this study, four mitochondrial and two nuclear markers were used to study genetic relationships within this complex. The phylogenetic analyses based on mtDNA gene trees constructed with ML and BI, and a species tree using *BEAST support three divergent clades in this region: the Valencia, Galera and Albacete/Murcia lineages. These three lineages were also corroborated in species delimitation analyses based on mtDNA using bPTP, mPTP, GMYC, ABGD and BAPS. Bayesian inference species delimitation method (BPP) based on both nuclear data and a combined dataset (mtDNA+nuclear) showed high posterior probabilities for these three SE lineages (≥ 0.94) and another Bayesian analysis (STACEY) based on combined dataset recovered the same three groups in this region. Divergence time dating of the species tree provided an estimated divergence of the Galera lineage from the other SE group (*P. vaucheri*, (Albacete/Murcia, Valencia)) at 12.48 Ma. During this period, the Betic–Rifian arc was isolated, which could have caused the isolation of the Galera form distributed to the south of the Betic Corridor. Although lizards from the Albacete/Murcia and Galera lineage are morphologically similar, they clearly represent distinct genetic lineages. The noteworthy separation of the Galera lineage enables us to conclude that this lineage must be considered as a new full species.

Key words: South-eastern, Iberian Peninsula, mtDNA, nuclear genes, *Podarcis*, phylogenetics

Running title: Multilocus analysis in SE Iberian Wall lizards

Introduction

Morphological and genetic studies spanning the last 25 years have attempted to clarify relationships both within and between lineages of the major lacertid lizard genus *Podarcis* from the Iberian Peninsula and North African. These studies reported that all *Podarcis* that inhabit this region, with the exception of *Podarcis muralis* (Laurenti, 1768), form a monophyletic group (Carranza et al., 2004b; Arnold et al., 2007) that is generally referred to as the *Podarcis hispanicus* (Steindachner, 1870) species complex.

Currently, seven genetically distinct lineages within this complex have been raised to species level: *P. guadarramae*, *P. virescens*, *P. liolepis*, *P. bocagei*, *P. carbonelli*, *P. vaucheri* and the nominal taxon *P. hispanicus* (Caeiro-Dias et al., 2018). The description of some of these species has been given in different works, so Harris & Sa-Sousa (2002) found molecular differences between two morphotypes (named types 1 and 2) from Western Iberia that were later described as two full species (Geniez et al., 2014): *Podarcis guadarramae* and *Podarcis virescens*, respectively. Moreover, they also divided *P. guadarramae* into two subspecies: *Podarcis guadarramae guadarramae* and *Podarcis guadarramae lusitanicus*. Harris & Sa-Sousa (2002), also described a further morphotype (type 3), that was later elevated to species rank as *Podarcis liolepis* (Renoult et al., 2010). Previously, other Iberian *Podarcis* were raised to species level, including *Podarcis bocagei* (Lopez-Seoane, 1885), *Podarcis carbonelli* (Pérez-Mellado, 1981) and *Podarcis vaucheri* (Busack et al., 2005).

The nominal taxon within the species complex *P. hispanicus* (Steindachner, 1870) was described as from the south-east (SE) of the Iberian Peninsula (Geniez et al., 2007). Within this group, Pinho et al. (2006) described a new mtDNA lineage from Galera locality, placed in the Baza Depression of SE Spain, represented by only a single specimen. This mtDNA lineage and that of *P. liolepis* clustered separately to other members of the species complex. Later work using allozyme markers (Pinho et al., 2007) largely corroborated the existence of a differentiated group in Galera area. In these first studies, monophyly of the Galera specimens with respect to other members of the species complex could not be addressed, due to the inclusion of individuals from a single location. Later genealogies based on two nuclear introns (Pinho et al., 2008) did not support *Podarcis* from Galera as a monophyletic group and pointed to nuclear gene flow between the Galera and other lineages identified by mtDNA analyses, where they are in sympatry, at least across parts of their distributions. Renoult et al. (2009) identified three evolutionary lineages (*P. virescens*, *P. liolepis* and *P. hispanicus*) in the east of Iberian Peninsula from morphological characters and nuclear loci, while their analysis of mtDNA revealed four lineages (*P. virescens*, *P. liolepis*, *P. hispanicus* from Galera and *P. hispanicus* from Valencia) suggesting an ancient introgression.

Other studies added to the Galera (Pinho et al., 2006) and Valencia (Renoult et al., 2009) forms, an additional mitochondrial lineage detected in SE Spain from the Albacete/Murcia area. The Valencia and Albacete/Murcia populations appear to comprise sister mitochondrial lineages, which together represent a sister group to all North African lineages, from which they diverged 6.99—9.44 Ma (Kaliontzopoulou et al., 2011). In that study a second individual from the Galera lineage was included and the results indicated that this mtDNA lineage clustered with those from *P. liolepis*, in agreement with Pinho et al. (2006) and unlike the results found by Renoult et al. (2009) which established that *P. liolepis* and *P. hispanicus* from Galera were two clearly independent groups.

The cluster composed by *P. liolepis* and the Galera lineage was estimated to have diverged from other members of the species complex 9.44—13.94 Ma (Kaliontzopoulou et al., 2011). All these studies trying to establish the phylogeny of *Podarcis* from the SE (Galera, Valencia and Albacete/Murcia lineages) are based on a very low number of individuals for each lineage. Consequently, the phylogenetic relationships between these groups remain unsolved and require a re-examination based on more extensive information.

For many years, the designation of mitochondrial lineages within the *P. hispanicus* complex have been identified solely by numbers and has coexisted with numerous systematic proposals for these lineages (Geniez et al., 2007, 2014). Despite the large number of studies carried out on this species complex, the phylogenetic relationships within the complex are not well established and there could be undiscovered independent lineages. The adequate assignment of potentially new species is a valuable instrument for conservation (Geniez et al., 2007, 2014; Renoult et al., 2010).

In this paper, we investigate the *P. hispanicus* complex with the main aim of establishing the phylogenetic relationships within the SE forms of *Podarcis* (Valencia, Galera and Albacete/Murcia lineage) and between them and other *Podarcis* from the Iberian Peninsula and North Africa. This is achieved through analyses of mtDNA, nuclear DNA and morphology. These analyses will contribute to knowledge of the evolutionary history and taxonomy of Iberian *Podarcis*, providing deeper biogeographical insights and important information for conservation bodies.

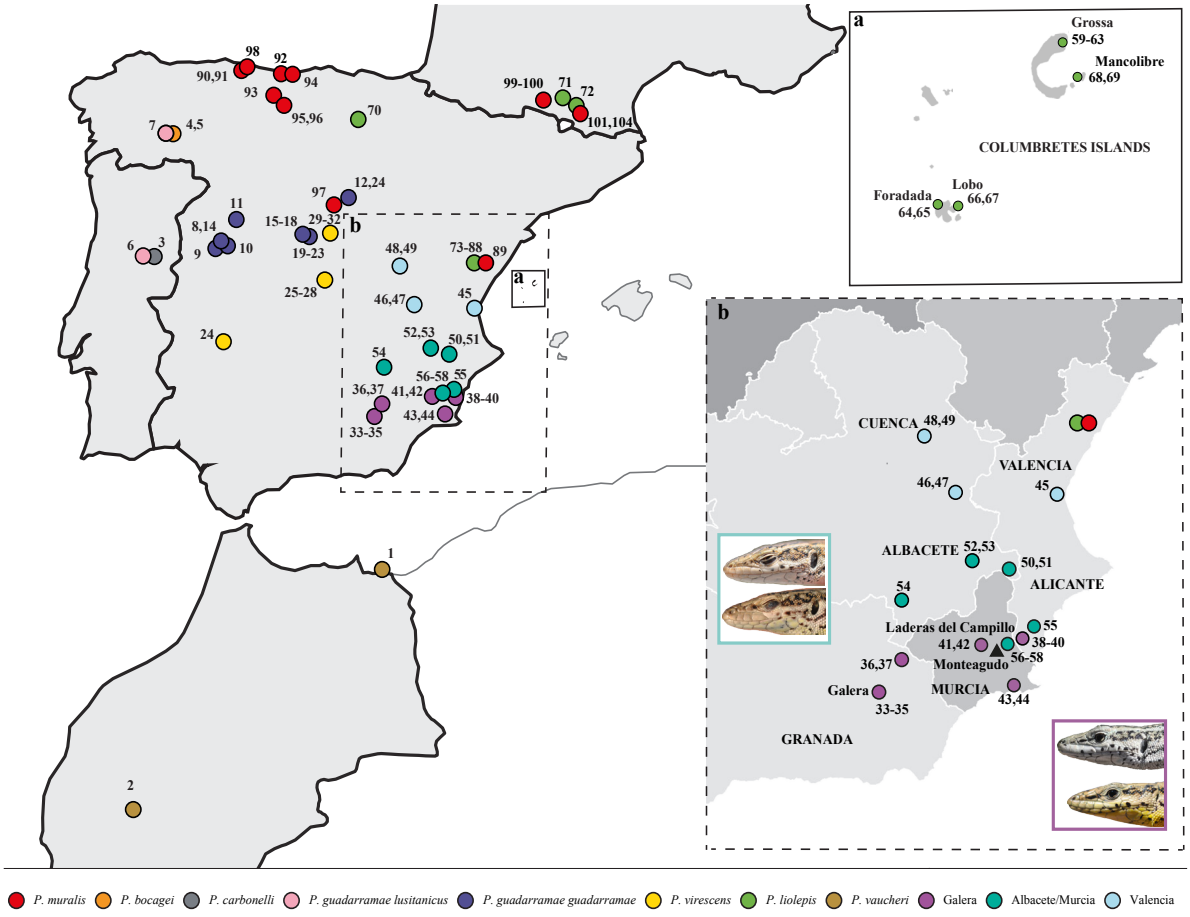


Figure 1. Locations of all *Podarcis* samples from the Iberian Peninsula and North Africa used in this study. The colors correspond to the different species indicated in the legend below and the numbers correspond to those in Supplementary Table 1. The black triangle marks the locality type of *Podarcis hispanicus sensu stricto* (Monteagudo, Murcia).

Material and methods

Sampling

In total, 104 *Podarcis* individuals from the Iberian Peninsula and North African region were captured (Supplementary Table 1 and Figure 1). Lizards were caught by careful noosing in their natural habitats with the specific permits delivered by the competent body in each locality. All morphological measurements were taken in situ and 1 cm of the tail tip was removed and stored in 100% ethanol. All lizards were released at their capture site.

Because the distribution of different lineages of *P. hispanicus* complex is not well-known in the SE Iberian Peninsula (see, for example, Caeiro-Dias et al., 2018 and references therein), we sampled some of the localities used by previous authors, where particular lineages were detected and described. In addition, we tried to include new localities within the expected range of each lineage.

Similar to previous authors (Geniez et al., 2007), we intensively searched the restricted type locality of *P. hispanicus* (Steindachner, 1870), Monteagudo, close to Murcia town, twice in 2019. Despite suitable climatic conditions, we did not find any *Podarcis* at the type locality but did detect a small population at Laderas del Campillo, 4.5 km north from Monteagudo (Figure 1). To our knowledge, this is the closest location to the type locality of *P. hispanicus sensu stricto* (Geniez et al., 2007). Lizards from Galera (Granada) were captured with a Permit of Scientific Capture from the Consellería de Agricultura, Ganadería, Pesca y Desarrollo Sostenible of Junta de Andalucía (permit No: 201999900530233 issued on 24/07/2019).

DNA extraction, amplification and sequencing

A standard phenol-chloroform protocol was used for DNA extraction (González et al., 1996). The following four non-overlapping mtDNA fragments were obtained for each specimen: i) partial 12S rRNA (360 bp), ii) partial control region (CR) (476 bp), iii) two partial fragments of cytochrome b (CYTB) (306 bp and 483 bp, respectively) and iv) two partial subunits of the NADH dehydrogenase gene and associated tRNAs (referred to as ND1 (48 bp), ND2 (415 bp), $tRNA_{Ile}$, $tRNA_{Gln}$, and $tRNA_{Met}$ (213 bp)). Two partial nuclear genes were amplified and sequenced: (i) melanocortin 1 receptor gene (MC1R) (663 bp) and ii) recombination activating gene 1 (RAG1) (939 bp) (Supplementary Table 1). Primers and amplification conditions are the same as those used in our previous studies of *Podarcis* (Buades et al., 2013; Rodriguez et al., 2013, 2014, 2017a; Terrasa et al., 2009a). Both strands of the PCR products were sequenced on an automated ABI 3130 sequencer (Applied Biosystems, Foster City, CA, USA) using a BigDye® Terminator v. 3.1 Cycle sequencing kit (Applied Biosystems) and edited using CodonCode Aligner software (CodonCode Corporation, Dedham, MA, USA). Nuclear data were phased using the PHASE algorithm (Stephens et al., 2001) within DnaSP v.6 (Librado & Rozas, 2009). Eighty-five sequences that had been published by previous studies were also used (Supplementary Table 1).

Phylogenetic analyses

We identified 88 unique haplotypes within the concatenated mitochondrial DNA dataset (2,301 bp) from the *Podarcis* specimens with DnaSP v.6 (Librado & Rozas, 2009). Individuals from the Balearic Islands were included as outgroups (five *Podarcis lilfordi* and three *Podarcis pityusensis*). Sequences were aligned in the MAFFT v7.423 online server (Katoh & Toh, 2008) using the iterative refinement method (FFT-NS-i). Best-fit nucleotide substitution models and partitioning scheme were chosen simultaneously using PartitionFinder v2.1.1 (Lanfear et al., 2016) under the Akaike Information Criterion (AIC). The partitioning schemes were defined manually (by gene and by codon), with branch lengths of alternative partitions "unlinked" to search for the best-fit scheme. The proportion of invariable sites (I) parameter was discarded if the favoured model incorporated both the I and the Gamma site rate heterogeneity (G) parameters as simultaneous use of these parameters can have undesirable effects (Yang, 1993).

We performed phylogenetic analyses using Maximum Likelihood (ML) and Bayesian Inference (BI) methods. Maximum likelihood analyses were performed using IQ-TREE version 1.6.10 (Nguyen et al., 2014). We applied the partitions and the best-fit substitution model and performed 10^6 bootstrap replicates based on the ultrafast bootstrap approximation (UFBoot) (Minh et al., 2013; Hoang et al., 2017) for statistical support.

Bayesian analyses were performed with MrBayes 3.2.6 (Ronquist et al., 2012). MCMC chain lengths were 10^7 generations with a sampling frequency of 10^3 generations. We used two simultaneous runs of three hot and one cold chain each. Convergence was confirmed by examining the stationarity of the log likelihood (lnL) values of the sampled trees and the observation of average standard deviations of the split frequencies being <0.01. Run characteristics such as effective sample sizes (ESS) were also assessed in Tracer v1.7 (Rambaut et al., 2018). The fifty-percent majority-rule consensus tree was summarized using sumt command with the first 25% of each run discarded as burn-in. The resulting phylogenetic tree was visualized and edited using Figtree v1.4.2 (Rambaut, 2014).

Haplotype networks were constructed for each phased nuclear locus using the Population Analysis with Reticulate Trees (PopART, http://popart.otago.ac.nz) (Leigh & Bryant, 2015) with the TCS method (Clement et al., 2000).

Species tree and divergence times estimates

A standard likelihood ratio test of the molecular clock was performed for both mtDNA and nuclear datasets in MEGA7 (Kumar et al., 2016). This test reliably informs whether a strict or relaxed clock model is most suitable for divergence time dating (Brown & Yang, 2011).

The species tree approach that is implemented in *BEAST (Heled & Drummond, 2010) was used in an attempt to simultaneously infer the phylogenetic relationships and divergence times between the different lineages of the Iberian and North African *Podarcis* based on: i) all mtDNA sequences dataset (108 individuals) and ii) both mtDNA and the phased nuclear loci (mtDNA+nuclear) (RAG1: 96 individuals and MC1R: 103 individuals). For mtDNA analyses, the species tree was calibrated using a mean split time of 5.3250 Ma with relatively little uncertainty around this estimate, replicating the calibration in Rodriguez et al. (2013).

The calibration was specified from a lognormal distribution with mean 1.6724 and standard deviation 0.002 (the central 95% of this distribution ranges from 5.304-5.346, in real space). This calibration is based on knowledge of the timing of the end of the Messinian salinity crisis (5.33 Ma) and the very rapid refilling of the Mediterranean basin that would have separated the two Balearic island *Podarcis* (i.e., *P. lilfordi* and *P. pityusensis*; see Brown et al., 2008 and references therein). Three partitions were assigned as: 1) 12S rRNA, CR, all tRNAs 2) CYTB/ND1/ND2 1st + 2nd codon position, and 3) CYTB/ND1/ND2 3rd codon position. Evolutionary models were the same as those used for MrBayes. The *BEAST MCMC sampler was run twice for $5x10^8$ generations, with one step per 50,000 being sampled. A relaxed log normal clock model was specified since the molecular clock likelihood ratio test indicated rate variation across the gene trees. A coalescent Yule speciation process was used for the tree prior. For mtDNA+nuclear analyses, we used the same calibration described for the mtDNA-only analyses. The same DNA substitution model was used for the three mtDNA partitions and the JC69 model was used for the two nuclear loci. Tracer v1.7 (Rambaut et al., 2018), was used to check for convergence. Posterior trees were combined to obtain the tree with the maximum sum of posterior clade probabilities using mean heights for node annotation.

Genetic structure and species delimitation analyses

Two tree-based (bPTP, mPTP and GMYC) and one distance-based (ABGD) species delimitation methods were performed on the concatenated mitochondrial haplotypes dataset. bPTP (Zhang et al., 2013b) analyses were performed using the online server (https://species.h-its.org/) and the ML tree from IQ-TREE. We ran 500,000 generations with a thinning of 500 and a burn-in of 0.1, then assessed convergence visually using the MCMC iteration vs. log-likelihood plot automatically generated. Next, we applied the mPTP method (Kapli et al., 2017) using the ML tree from IQ-TREE. We performed two simultaneous Markov Chain Monte Carlo (MCMC) runs of 100,000,000 steps, sampling every 10,000 steps. Convergence was confirmed with the likelihood plot of the combined runs and the Average Standard Deviation of Delimitation Support Values (0.000009), which indicate convergence on the same distribution as it approaches to zero. The -multi option was used to allow differences in rates of coalescence among species. Finally, we incorporated the single threshold GMYC (Pons et al., 2006; Fujisawa & Barraclough, 2013) model. This model is based on an ultrametric phylogenetic tree and uses differences in branching rates to infer species delimitation. The input tree was generated in BEAST v2.6 (Bouckaert et al., 2019) under a relaxed clock and Yule Model tree prior. The same partitioning model used for the ML and BI trees was used as estimated using AIC in PartitionFinder v2.1.1 (Lanfear et al., 2016). Analyses were run for 200 million generations, sampling every 2,000 generations. Convergence and mixing were monitored in Tracer v1.7 (Rambaut et al., 2018), and ESS values >200 indicated adequate sampling of the posterior. TreeAnotator within BEAST was used to create a tree with the maximum sum of posterior clade probabilities using mean heights for node annotation and a 10% of burn-in. The GMYC analysis was conducted using the single-threshold method in the *splits* package on R v. 3.6.1.

Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012) uses pairwise genetic distances to determine the differences between intra- and interspecific divergence and establishes the number of different species based on those differences. ABGD was performed using the online tool (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb) with default values for the prior intraspecific divergences (Pmin 0.001, Pmax 0.1, steps 10), relative gap width (1.5), distance distribution (20). Results were compared using both JC69 and K80 models.

Structural analysis was performed with the Bayesian model-based clustering algorithm implemented in BAPS 6.0 (Corander & Tang, 2007) using the mtDNA haplotypes alignment. The method was performed using clustering with linked loci and codon linkage model. We ran BAPS for values of k ranging from one to fifteen, performing three replicates for each value of k.

Bayesian Phylogenetics and Phylogeography (BPP v4.0) (Yang & Rannala, 2010) analysis was performed on two different datasets 1) two phased nuclear loci and 2) mtDNA + two phased nuclear loci. We also included Balearic *Podarcis* in the analysis as the outgroup. BPP is a robust means of inferring species from recently diverged lineages using multilocus data (Rannala, 2015; Leaché et al., 2018) under the multispecies coalescent model (MSC) (Rannala & Yang, 2003). This Bayesian method assumes no gene flow among species and no recombination among loci and explains gene tree discordance by incomplete lineage sorting (ILS). BPP implements a reversible jump Markov chain Monte Carlo (rjMCMC) search to estimate the sum of posterior probability (PP) of all species delimitation models considered. The speciation models PP can be affected by the prior distributions chosen for the ancestral population size (θ) and root age (τ). We used a range of different prior scenarios (Table 1), including small population size (θ) and deep divergence (τ), or large population size and shallow divergence that favours more conservative models containing fewer species (Yang & Rannala, 2010).

We used the A11 model (speciesdelimitation=1 and speciestree=1), which jointly infers the species tree and the species delimitation (Rannala & Yang, 2017). The assignment of individuals to hypothesized species was based on the identified mtDNA lineages. The MCMC chain was run for 10^4 steps (following a burn-in of 2,000 steps), sampled every 25 steps. Each analysis was run three times to confirm that they converged on the same posterior.

Finally, we used STACEY v1.8.0 (Jones, 2017), a Bayesian multispecies coalescent method implemented in BEAST v2.5 (Bouckaert et al., 2019) that allows inference of species delimitation and species phylogeny. The author of this analysis prefers the term 'cluster' over 'species' (Jones, 2017) and so we use both terms when discussing this analysis. STACEY provided a parallel analysis to that in BPP and so was particularly useful to ensure the robustness of the species delimitation results. STACEY was preferred over a similar approach within *BEAST (Heled & Drummond, 2010; Grummer et al., 2014) because it provided better convergence of the MCMC chain. It differs from BPP in several ways, including the implementation of a Birth-Death-Collapse model, which eliminates the need for a rjMCMC algorithm, and use of different population size parameters which are integrated out. As for BPP analyses, we used the three loci comprised mtDNA and the two phased nuclear loci with the JC69 substitution model and a strict clock specified for each. Two independent runs of 450 million generations of the MCMC chains were performed sampling every 5,000 steps. Convergence was checked in Tracer 1.7 (Rambaut et al., 2018) and posterior trees from both runs were combined (the first 20% of trees from each run were removed as burn-in). The resulting 144,000 species trees were processed in SpeciesDelimitationAnalyser (Jones, 2017) with a collapse height of 0.0001 (the same value used in STACEY analysis) and default similarity cut-off (0.9). Posterior trees were also combined to obtain a maximum sum of clade credibilities tree.

Morphological analyses

To avoid any confusion regarding their lineage assignation, our morphometric analyses were restricted to individuals that we personally generated sequence data for. Thus, we analyzed a rather small sample of lizards (25 individuals from Galera lineage and 12 belonging to Albacete/Murcia lineage).

Our aim was only to show general similarities and differences between the two lineages of Albacete/Murcia and Galera. A more thorough analysis will be subsequently carried out with a larger sample of individuals from the entire Iberian *Podarcis* species complex.

Six body dimensions, as well as body mass (Weight), were included in this study: snout-vent length (SVL), intact tail length (TL), pileus length (PL), head width (HW), head height (HH) and left hind limb length (HLL). All measurements were made with a digital calliper to the nearest 0.01 mm, except for SVL and intact tail length, which was measured with a steel rule to the nearest 1 mm. Weight was obtained with a spring scale Pesola®. Six scalation characters were recorded: gularia, collaria, dorsalia, ventralia, femoralia, and left fourth digit lamellae (see Pérez-Mellado & Gosá, 1988) for methodological details of body measurements and scalation counts). Not all characters could be recorded from all individuals. Due to sexual dimorphism, males and females were analysed separately. Raw or log-transformed data were checked for normality (Shapiro-Wilk test) and homogeneity of variances (Fligner test) prior to statistical comparisons (one-way ANOVA). If assumptions for the use of parametric techniques were not met, we employed non-parametric equivalents. All analyses were done within R (R Core Team, 2018). In addition, we studied colour and design variation in our samples, employing the same criteria as Geniez et al. (2007) in their diagnostic descriptions. Colour descriptions were completed with the assignment of colour codes to the following body parts: background dorsum, pileus, flanks, belly, gular region, blue ocelli in outer ventral scales, if present, dorsolateral stripes, dorsal side of the tail and ventral side of the tail. Colour codes were assigned according with the catalogue of Köhler (2012).

Results

Phylogenetic analysis

PartitionFinder v2.1.1 identified three partitions with the following substitution models: non-coding fragments [GTR+I+G], 1st and 2nd codon of coding regions [HKY+I+G] and 3rd codon of coding regions [GTR+I+G] (analyses were carried out with these models but without the I parameter, as discussed in the Material and Methods). ML and BI trees clustered the individuals into four major mitochondrial clades within the Iberian Peninsula and North African samples, corresponding to the following taxa/areas: group 1 (monophyletic) only includes *P. muralis*; the other groups are polyphyletic including group 2: *P. carbonelli*, *P. virescens*, *P. g. lusitanicus*, *P. bocagei* and *P. g. guadarramae*, group 3: *P. vaucheri*, Galera lineage, Valencia lineage and Albacete/Murcia lineage, and group 4: *P. liolepis* (Figure 2). The separation between groups 3 and 4 presents low support (Posterior Probability < 50; Bootstrap Support < 40). Divergence of the different SE mitochondrial lineages (Galera, Albacete/Murcia and Valencia) show high support (PP≥0.99; BS≥79). The results show Valencia and Albacete/Murcia as sister lineages to the North African form (*P. vaucheri*), while the entire group (*P. vaucheri*, (Albacete/Murcia, Valencia)) is a sister lineage to the Galera lineage.

Networks based on phased nuclear loci (Figure 3), show less structured interrelations between different *Podarcis* populations. *Podarcis muralis* and *P. vaucheri* are the only groups that share no haplotypes with any other *Podarcis* populations. The Galera lineage presents two shared haplotypes and nine species-specific haplotypes in the RAG1 network, and one shared haplotype and five unique haplotypes in the MC1R network. These haplotypes were shared with geographically close groups (*P. virescens*, *P. liolepis*, Valencia and Albacete/Murcia lineages), except for *P. g. lusitanicus*, indicating a common haplotype among these populations with posterior differentiation.

The striking number of different haplotypes found in *P. liolepis* demonstrates the high genetic diversity present in this group and could be an indicator that its distribution range has not been fully studied genetically.

Species tree and divergence time estimates

The mtDNA species tree (Figure 4) provided a posterior mean for the divergence of the Iberian and North African group with respect to the monophyletic group *P. muralis* at 15.60 Ma (95% highest posterior density, HPD: 22.31–10.71 Ma), and that for the divergence of Galera from the clade containing (*P. vaucheri*, (Albacete/Murcia, Valencia)) at 12.48 Ma (95% HPD: 18.27–7.93 Ma). The Valencia and Albacete/Murcia lineage divergence appeared to be more recent at 7.11 Ma (95% HPD: 11.75–1.87 Ma). The split between *P. liolepis*, and the remaining lineages from west Iberia would have taken place during the middle Miocene, in the late Serravallian (12.58 Ma, 95% HPD: 18.53-7.40 Ma), as also observed for the divergence of the Galera lineage. Despite the use of simpler models with fewer parameters, the mtDNA+nuclear analyses did not show repeatable convergence on the same posterior and, thus, the results are not presented here.

Genetic structure and species delimitation analysis

The different species delimitation methods (bPTP, mPTP, GMYC, ABGD and BAPS), based on mtDNA, returned different partition numbers, ranging from 9 to 22. All the methods corroborated the separation of the SE lineages (Valencia, Albacete/Murcia and Galera) (Figure 2).

Under five out of seven prior specifications for θ and τ, BPP analyses for both dataset nuclear and combined mtDNA+nuclear loci provided the greatest support for the delimitation of 12 species (Table 1). The analyses in which the parameters favoured a moderate populations size (θ: InvG (3, 0.02)) and divergence time (τ0: InvG (3, 0.02)) provided the highest posterior probability (0.91 and 0.95, respectively) for 12 species. This contrasts with analyses that favoured a larger population size (θ: InvG (3, 0.2)) and a deep/moderate divergence time (τ0 InvG (3, 0.2)) or (τ0 InvG (3, 0.02)), which provided the greatest support for 11 species (0.520 and 0.62, respectively). *Podarcis bocagei* and *P. carbonelli* are the two populations that are not clearly defined as separate species (Table 1 and Figure 2). In terms of species delimitation, all prior combinations showed strong individual species support (≥ 0.94) for the Galera, Albacete/Murcia and Valencia lineages (Table 1). Nonetheless, the inferred species tree topologies tended to vary among models, and even (to a lesser extent) showed some minor differences between runs for a given prior combination. Posterior support for nodes within the BPP species trees based on both nuclear data and mtDNA+nuclear combined data was generally low, except for *P. muralis* and *P. vaucheri*, and the topology differed from that described by phylogenetic trees based on the mtDNA concatenated dataset.

Species delimitation analysis performed with STACEY provided similar results to those obtained using BPP and attributed highest posterior probabilities to 13 clusters (0.96). In this analysis, an extra group was found corresponding to the separation of the outgroup into the two recognised species from the Balearic islands (*P. lilfordi* and *P. pityusensis*). Nonetheless, the three SE lineages from the Iberian Peninsula are confirmed to represent independently evolving lineages. Also similar to BPP analyses, support values were low for individual internal nodes within the species tree, except for the divergence of *P. vaucheri* from the rest of species complex.

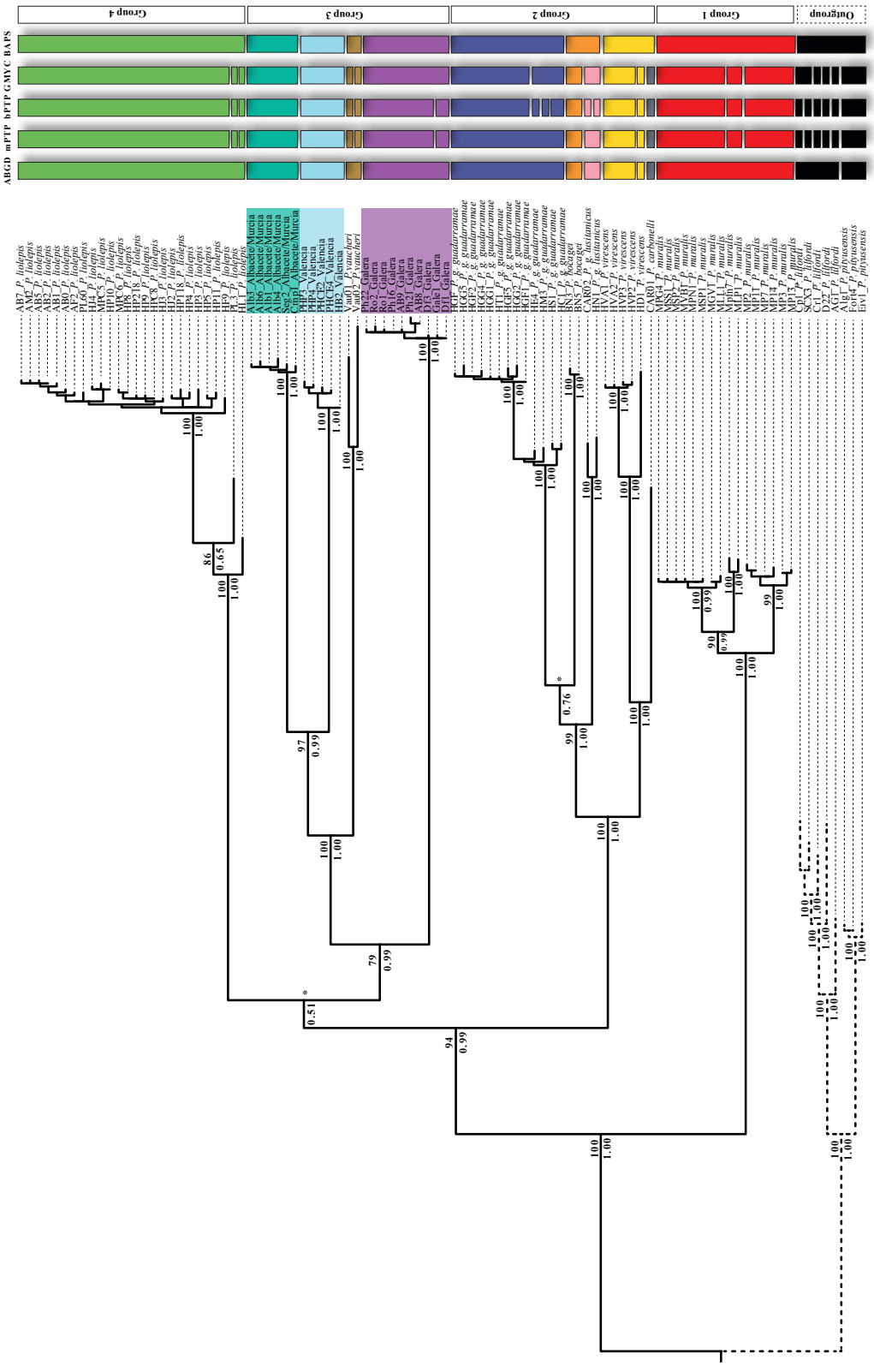


Figure 2. Gene tree based on Maximum Likelihood for mitochondrial data showing different scenarios of species delimitation (ABGD, mPTP, bPTP, GMYC, BAPS) between Iberian and North African *Podarcis* populations, using the Balearic clade as an outgroup. The numbers above branches correspond to bootstrap support and numbers below branches are posterior probabilities from Bayesian analysis. Support values for nodes with ≤ 50% support are not included. Asterisks indicate branches where different analyses produced different topology. Group 1: *P. muralis;* Group 2: *P. bocagei, P. carbonelli, P. g. guadarramae, P. g. lusitanicus, P. virescens;* Group 3: *P. vaucheri,* Galera lineage, Albacete/Murcia lineage, Valencia lineage; Group 4: *P. liolepis.*

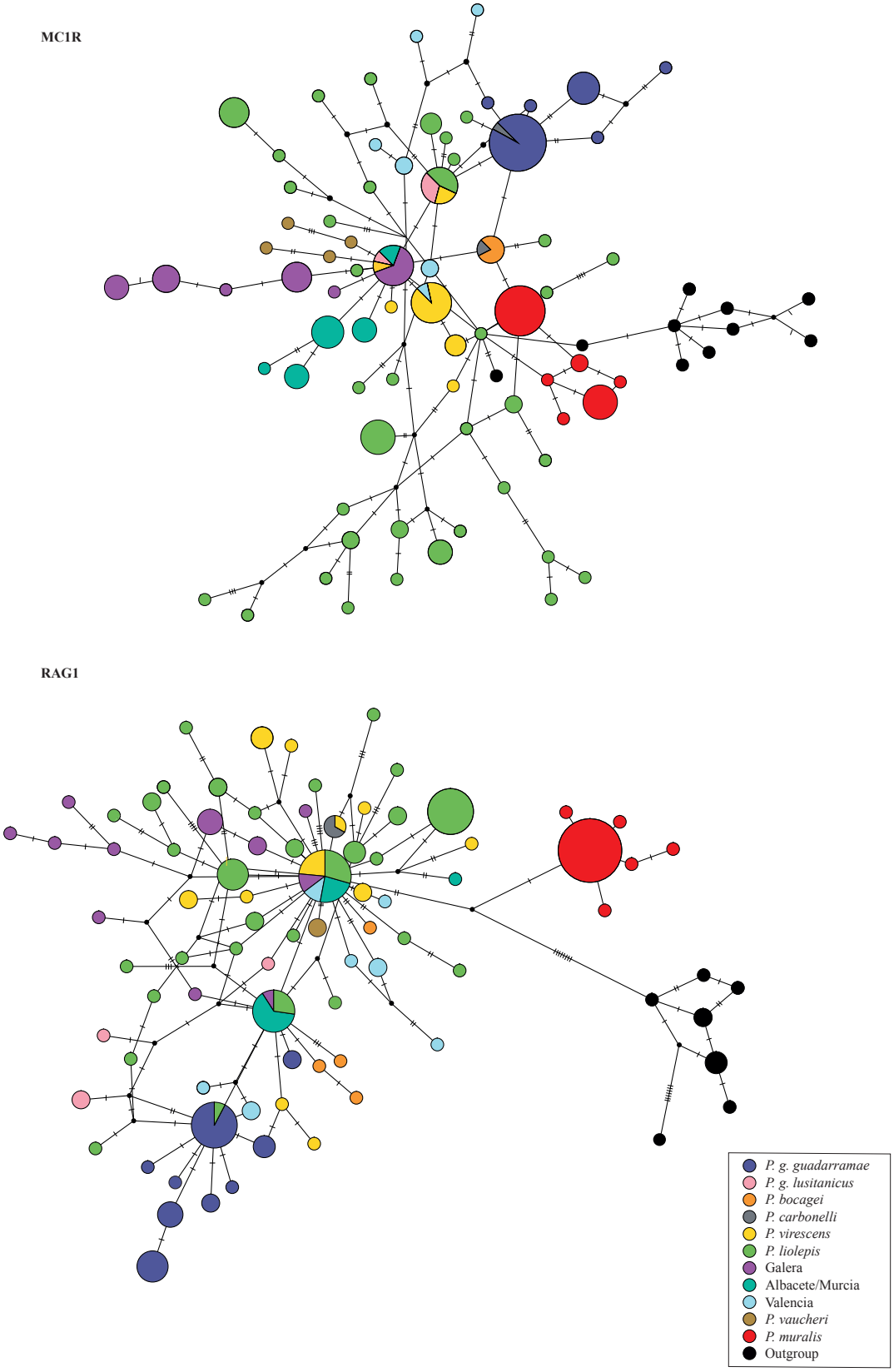


Figure 3. TCS haplotype networks of Iberian and North African *Podarcis* nuclear loci for Melanocortin receptor 1 (MC1R) and Recombination activating gene 1 (RAG1). Hatch marks between black dots represent one mutational step, haplotypes circle area is proportional to the number of individuals and the colour identifies the species.

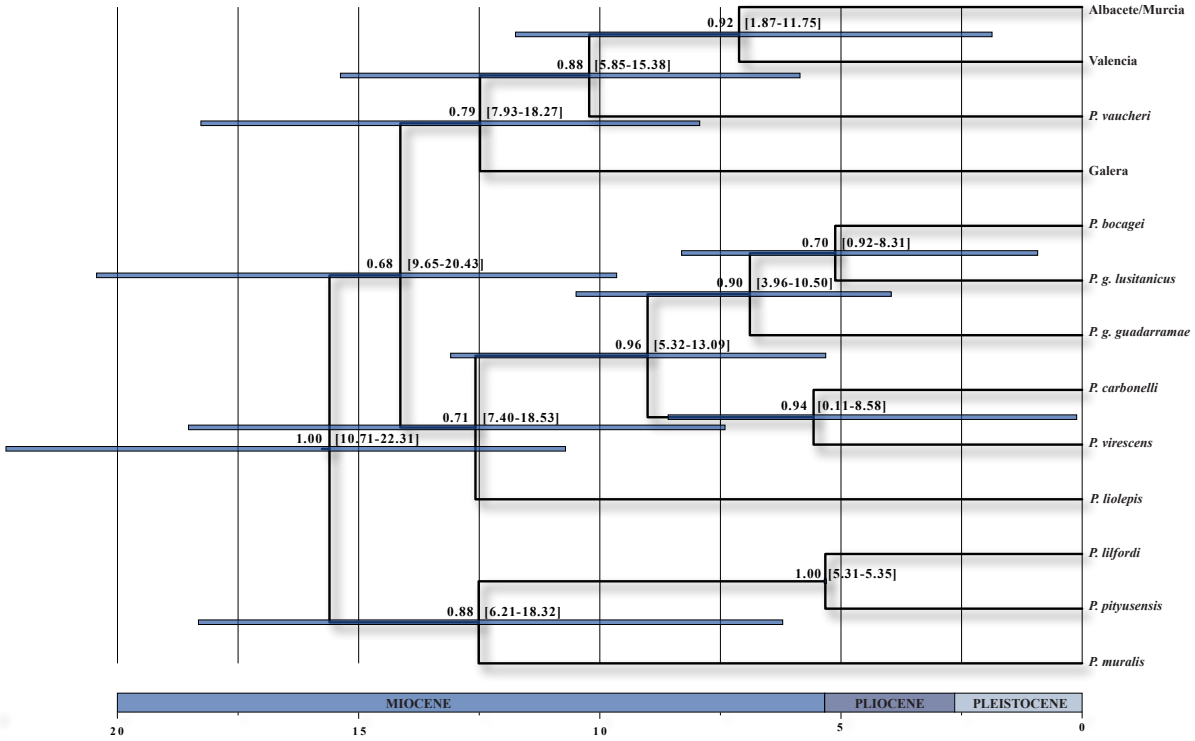


Figure 4. Species tree with estimated divergence time based on mitochondrial data (2,301 bp of 12S, CR, CYTB and NADH fragments) resolved from *BEAST analyses. The divergence times were estimated using the separation of the Balearic *Podarcis* clade as a calibration. Horizontal bars and numbers at the right of nodes (Ma) indicate a 95% credible interval of divergence and posterior probability values are shown to the left.

Morphological analysis

In the SE Iberian Peninsula, both groups of lizards from the Albacete/Murcia and Galera lineages are characterized by a very small body size (SVL and Weight) in comparison to the remaining recognized species of Iberian *Podarcis* (Kaliontzopoulou et al., 2012). In both lineages, we found an SVL under 53 mm (Supplementary Table 2). Body size is statistically similar in the Albacete/Murcia and Galera lizards (Supplementary Table 2, adult males: one-way ANOVA, $F_{1,22} = 1.816$, p= 0.192, adult females: $F_{1,11} = 0.173$, p= 0.686) as is body mass (males: $F_{1,19} = 0.0128$, p= 0.991, females: $F_{1,10} = 3.703$, p= 0.083). The number of lizards with an intact tail precluded any comparison between lineages (Supplementary Table 2). For head measurements, only head width showed significant differences between males of Albacete/Murcia and Galera ($F_{1,22} = 9.541$, p= 0.0054) but not between females ($F_{1,11} = 2.041$, p=0.181). The remaining head measurements were similar in both lineages (pileus length, males: $F_{1,22} = 1.658$, p= 0.211, females: $F_{1,10}$= 0.06, p= 0.811; head height, males: $F_{1,22} = 1.832$, p= 0.19, females: $F_{1,11} = 1.53$, p= 0.242). Hind limb length was also similar in both lineages (males: $F_{1,22} = 3.1$, p= 0.0922, females: W=6, p=0.106).

With regard to scalation traits, the number of subdigital lamellae under the 4th toe is similar in both lineages (males: $F_{1,21}$= 0.43, p=0.519, females: $F_{1,7} = 1.485$, p=0.262). There is a greater number of femoral pores in males from the Albacete/Murcia lineage (Supplementary Table 2, $F_{1,22} = 16.48$, p= 0.00052) but not in females ($F_{1,10} = 0.561$, p= 0.471).

Also, the number of gular scales is greater in Albacete/Murcia males (Supplementary Table 2, $F_{1,22}$ = 27.02, p= $3.26x10^{-5}$) but not in females ($F_{1,10}$ = 0.944, p= 0.354). We did not find differences in the number of dorsal scales (males: $F_{1,21}$ = 0.19, p= 0.668, females: $F_{1,9}$ = 2.065, p= 0.185) nor in collaria (males: $F_{1,22}$ = 0.132, p= 0.72, females: $F_{1,9}$ = 1.205, p= 0.301). Finally, the number of ventral scales is significantly higher in Galera lizards (Supplementary Table 2, males: Mann-Whitney test, W= 23.5, p= 0.0123, females: $F_{1,9}$ = 7.333, p= 0.024).

Lizards from both lineages of Albacete/Murcia and Galera are small dorsally brownish or greyish lizards. The general appearance of the lizards from both lineages is very similar (Supplementary Figure 1 and 2). In the Galera lineage, we observed dorsal colours from Drab (code 19 of Köhler, 2012) to Light Drab (269) and, especially, Olive-Brown (278) and Brownish Olive (276) (Supplementary Table 4). The dorsal background of lizards from the Albacete/Murcia lineage is mainly Drab (19), Ground Cinnamon (270) or Light Drab (269). Thus, in the Albacete/Murcia lineage we can observe olive nuances that, apparently, are absent in the Galera lineage. A similar pattern was observed in pileus colour (Supplementary Table 4). In the Galera lineage, bellies are mainly Smoky White (261) or Light Buff (2), while in the Albacete/Murcia lineage bellies are Pale Buff (1) or Light Buff (2) (Supplementary Table 4).

Taking into account the same diagnostic characters employed by Geniez et al. (2007) to define the nominotypical taxon, *P. hispanicus*, we can see that almost all diagnostic features of colour and design can be found in both lineages. However, all these traits are better represented by the Albacete/Murcia lineage (Supplementary Table 3 and Supplementary Figure 2). A wider sampling of its geographic region is required to provide an in-depth morphological description of the Valencia lineage.

Taxonomic results

Geniez et al. (2007) carried out a morphological analysis of several lizards from the SE Iberian Peninsula, before the discovery of two new lineages: Albacete/Murcia and Valencia (Caeiro-Dias et al., 2018 and references therein). Consequently, it is possible that their sample contained lizards from different lineages. We do not know if this was the case, but from our results, lizards from the Albacete/Murcia lineage are closer to the nominotypical taxon, as defined by Geniez et al. (2007) (see Supplementary Table 3). In addition, at the closest locality to Monteagudo, Laderas del Campillo, we found lizards assigned to the Albacete/Murcia lineage. Thus, we propose that the Albacete/Murcia lineage could be considered the present-day representation of the nominotypical taxon, *Podarcis hispanicus sensu stricto*. In fact, the phylogenetic tree (Figure 2) shows that there is a monophyletic group formed by the Valencia lineage and *P. h. sensu stricto* (Albacete/Murcia lineage). This clade is the sister group to *P. vaucheri*. The Galera lineage is a monophyletic group, clearly separated from the other Eastern lineages of Iberian *Podarcis*. In line with this result and the arrangement of different lineages in the phylogeny of Iberian *Podarcis* (Table 1 and Figures 2 and 4), we can conclude that the Galera lineage represents a new full species: *P. galerai* sp. nov. The taxonomic arrangement of this lineage within the Iberian *Podarcis* complex and the description of the new species in comparison with the nominal taxon *P. hispanicus* is proposed below.

Table 1. Results from BPP analyses for each combination of priors based on phased nuclear loci only (first row) and on combined dataset mtDNA+nuclear loci (second row). The posterior probability (PP) values are averages from three independent runs of module A11. Abbreviations: Bal, Balearic outgroup; Gal, Galera lineage; Lio, *P. liolepis;* Alb, Albacete/Murcia lineage; Mur, *P. muralis;* Gua, *P. g. guadarramae*; Val, Valencia lineage; Lus, *P. g. lusitanicus;* Vir, *P. virescens*; Boc, *P. bocagei;* Car, *P. carbonelli;* Vau, *P. vaucheri.*

Priors			PP of each species														PP for number of species		
θ	τ0		P[[Bal]	P [Gal]	P [Lio]	P [Alb]	P [Mur]	P [Gua]	P [Val]	P [Lus]	P [Vir]	P [Boc]	P [Car]	P [Vau]	P [Boc-Car]	P [Vir-Car]	P [12]	P [11]	P [10]
G (3. 0.02)	G (3. 0.004)	nuclear	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.95	0.97	0.92	1.00	0.03	0.04	0.91	0.09	0
G (3. 0.02)	G (3. 0.004)	mt+nuclear	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.96	0.95	1.00	0.04	0.00	0.95	0.05	0
G (3. 0.02)	G (3. 0.2)	nuclear	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.99	0.90	0.94	0.83	1.00	0.06	0.10	0.82	0.18	0
G (3. 0.02)	G (3. 0.2)	mt+nuclear	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.89	0.89	1.00	0.11	0	0.89	0.11	0
G (3. 0.02)	G (3.0.02)	nuclear	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.95	0.97	0.91	1.00	0.03	0.05	0.91	0.09	0
G (3. 0.02)	G (3.0.02)	mt+nuclear	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.95	0.95	1.00	0.05	0	0.95	0.05	0
G (3. 0.2)	G (3.0.02)	nuclear	1.00	1.00	1.00	0.99	1.00	1.00	0.96	0.71	0.61	0.62	0.31	0.85	0.27	0.24	0.22	0.52	0.23
G (3. 0.2)	G (3.0.02)	mt+nuclear	1.00	1.00	1.00	1.00	1.00	1.00	0.99	0.87	0.77	0.59	0.38	0.92	0.37	0.18	0.34	0.57	0.09
G (3. 0.2)	G (3. 0.2)	nuclear	1.00	1.00	1.00	0.98	1.00	1.00	0.94	0.54	0.45	0.60	0.20	0.69	0.22	0.26	0.11	0.41	0.41
G (3. 0.2)	G (3. 0.2)	mt+nuclear	1.00	1.00	1.00	1.00	1.00	1.00	0.98	0.76	0.70	0.44	0.23	0.88	0.48	0.18	0.18	0.62	0.20
G (3. 0.002)	G (3. 0.004)	nuclear	1.00	1.00	1.00	1.00	1.00	1.00	0.97	1.00	0.93	0.93	0.86	1.00	0.07	0.05	0.85	0.15	0
G (3. 0.002)	G (3. 0.004)	mt+nuclear	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.99	0.96	0.95	1.00	0.04	0.01	0.95	0.05	0
G (3. 0.002)	G (3. 0.04)	nuclear	1.00	1.00	1.00	1.00	1.00	1.00	0.98	0.99	0.92	0.92	0.84	1.00	0.08	0.09	0.82	0.17	0
G (3. 0.002)	G (3. 0.04)	mt+nuclear	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.99	0.99	0.94	0.93	0.99	0.06	0.01	0.93	0.07	0

Taxonomy

Family LACERTIDAE

Genus *Podarcis Wagler*, 1830

Podarcis hispanicus Steindachner, 1870

Lacerta oxycephala var. *hispanica* Steindachner, 1870. S*itzungsber. Akad. Wiss. Wien, Math. Naturwiss. Kl.,* Abt.1, 62(8): 350.

Lacerta muralis steindachneri Bedriaga, 1886. *Abh. Senckenberg. Naturforsch. Ges*., 14(2): 256.

Podarcis [hispanicus] hispanicus Steindachner, 1870, Geniez et al., 2007. *Herpet. J*., 17:74.

Type locality. Monteagudo (not Monte Agudo), province of Murcia. *Terra typica restricta* Geniez et al. (2007).

Geniez et al. (2007) designed as the lectotype the specimen NMW 16088:1 (Naturhistorisches Museum Wien, Austria).

Diagnosis. Due to the coincidence of several morphometric traits of the lineages from Galera and Albacete/Murcia, the diagnosis of Geniez et al. (2007) is, in general, valid for both lineages, here recognized as separated species. A small lizard with a very flattened head (Supplementary Table 2). Generally, it lacks the masseteric plate. Dorsum brown. In most lizards, there is a continuation of light dorsolateral stripes and dark stripes along parietal scales of the head. Normally (more than 83% of individuals), vertebral line is present and in the majority of cases, bi or even trifurcated (Supplementary Table 3 and Supplementary Figure 2). Belly with white color, even if more than 30% of individuals can show a yellowish color, in some cases covering the base of the tail (Supplementary Figure 2). In 25% of individuals, blue ocelli are present in outer ventral scales. From 56 to 73 dorsal scales (Geniez et al., 2007, mention lizards with a minimum of 44 dorsal scales).

Podarcis galerai sp.nov.

Holotype. Herpetological collection (Colección Herpetológica de la Universidad de Salamanca, CHUS) of the Department of Animal Biology (University of Salamanca, Salamanca, Spain), CHUS01140319, holotype by present designation; adult male captured by Ana Pérez-Cembranos and Valentín Pérez-Mellado on 14th September 2019 in the village of Galera (Granada province, Spain).

Type locality. Galera, province of Granada (Spain).

Description of the holotype. Adult male (Supplementary Figure 1) with 48.5 mm of south-vent-length and 115 mm of its intact tail length, 11.67 mm of pileus length, 5.62 mm of pileus width, 5.29 mm of head height, 15.85 mm of front leg length, 26.42 mm of hindleg length and 11.67 mm of foot length. Scalation: 56 longitudinal rows of dorsal scales at mid-body (dorsalia), 25 gular scales (gularia), 10 collar scales (collaria), 21 transversal rows of ventral scales from the collar to the anal plate (ventralia), 14 femoral pores on left hindleg (femoralia), 15 femoral pores on right hindleg, 27 subdigital lamellae under the fourth toe, 6 submaxillary scales on each side, 8 supralabial scales on each side, 6 supraocular scales on each side and 10 supraciliary granules on each side. Coloration on the live animal: iris light brown. Pileus uniformly light brown. Dorsum brown, finely dotted.

The individual lacks a well-formed vertebral line, although there is an alignment of disconnected blackish points. Light brown on dorsolateral lines, well-marked and with sharp edges, with dark supra-dorsolateral bands formed by unconnected black spots. Dorsolateral stripes continue over the pileus. Tail greenish, clearly contrasted with the brown dorsum. Upper half of flanks dark brown, speckled with black. Lower half of flanks with light brown background, stained with dark brown. Ventral whitish-grayish. Gular area only with some black point in the lateral zones. Outer ventral scales with faded gray spots. Blue ocelli in all outer ventral scales. Submaxillary scales not pigmented (Supplementary Figure 1).

Etymology. The epithet *galerai* refers to the type locality: Galera, a village from northeastern Granada province (Spain).

Diagnosis. A small wall lizard with less than 53 mm of SVL (see Supplementary Table 2 for averages, maximum and minimum values of morphometric and scalations characters). Dorsal pattern and colour very variable among localities and even within a given population. Dorsum always light or dark brown, greyish or even reddish, never green. Light dorsolateral stripes that can continue or not over parietal plates of the head. Supra-dorsolateral stripes dark brown or black, present in almost all individuals. As in other Iberian *Podarcis*, light dorsolateral stripes are better defined in adult females. Upper half of flanks with a light brown spotted with small dark brown or black dashes, profusely reticulated with black spots or uniformly black, particularly in adult females. Lower half of flanks with a light brown sparsely spotted with brown or black small spots. Between upper and lower half of flanks, frequently there is a light brown stripe, again better defined in adult females. Vertebral line is present in only a half of individuals (Supplementary Table 2). When present, vertebral line is frequently bifurcated, especially on the upper half of dorsum. In a majority of adult individuals (Supplementary Table 2), belly is white or grey. However, a minor proportion of lizards shows a yellowish or even an orange belly (Supplementary Table 3 and Supplementary Figure 1). More than 40% of adult males have blue ocelli on outer ventral scales. Masseteric plate is absent. Juveniles, many adult females and even some adult males, can have a blue or greenish tail, sharply contrasted with brown dorsum.

Comparison with other species. Supplementary Table 3 shows the main differences between *P. hispanicus* and *P. galerai* sp. nov., comparing coloration and design traits of both species with descriptions by Geniez et al. (2007). Body size and general aspect of *P. hispanicus* and *P. galerai* sp. nov. are quite similar. Males of *P. galerai* sp. nov. show a wider head width and a lower number of femoral pores than *P. hispanicus*. Both sexes of *P. galerai* sp. nov. show a higher ventralia. In addition, in *P. galerai* sp. nov. we observe a more pointed snout and a more flattened head (Supplementary Figure 1). There are several individuals that lack a vertebral line. When present, vertebral line is bifurcated in less than a half of adult lizards (Supplementary Table 3 and see above the diagnosis of *P. galerai* sp. nov.). In *P. galerai* sp. nov., belly is normally white as in *P. hispanicus*, but there are individuals with yellowish or even orange bellies. In addition, in several adult males it is possible to observe blue ocelli on outer ventral scales, a trait only observed in a minority of *P. hispanicus* males.

Distribution and ecology. The geographical distribution of *P. hispanicus* and *P. galerai* sp. nov. is poorly known. According to our present-day data, *P. hispanicus* is approximately distributed from the latitude of Murcia town to the nort*h.* Apparently, *P. hispanicus* is today absent from its terra typica restricta, Monteagudo (Murcia province, Geniez et al., 2007 and pers. Obs.). We ignore the northern limit of this distribution, especially in relation to *Podarcis liolepis*. Similarly, the western limit remains to be clarified in relation to the geographical area occupied by *Podarcis virescens*.

Our westernmost locality was Cañada del Provencio, in Sierra de Alcaraz (Albacete province). From our survey, the easternmost locality of *P. hispanicus* would be Callosa de Segura (Alicante province). Unfortunately, we cannot resolve if lizards from Elche (Alicante) can be assigned to *P. hispanicus* (Renoult et al., 2009). *Podarcis galerai* sp. nov. is mainly present to the south of Murcia town, apparently reaching coastal areas of Almería province (between Dalias and Berja, Caeiro-Dias et al., 2018, from data of Renoult et al., 2009). To the east, *P. galerai* sp. nov. arrives to Embalse de la Pedrera, in Orihuela (Alicante province). To the west, it would be present 2 km to the north of Tíscar (Jaén province, Caeiro-Dias et al., 2018). Caravaca de la Cruz (Murcia province) would be the northernmost known locality of *P. galerai* sp. nov. (Kaliontzopoulou et al., 2011). It is clear, that we would need a deeper survey of geographical ranges of both species. Particularly interesting will be to study overlap areas of both lizard species. According with known localities, the altitudinal range of both species seems to be very similar (from 90 to 1180 m.a.s.l. in *P. hispanicus* and 120 to 1164 m in *P. galerai* sp. nov.). Both species are clearly saxicolous. As other Iberian species (i.e., *P. guadarramae* (Geniez et al., 2014 and references therein)), *P. hispanicus* and *P. galerai* sp. nov. are mainly adapted to rupicolous habitats. *P. hispanicus* is found in arid landscapes close to Murcia town (Laderas del Campillo), occupying ruins and artificial walls with crevices. It is also present in rocky outcrops inside pine forests (Cañada del Provencio). We also found lizards isolated in small accumulations of stacked stones that were removed from cultivated areas (Montealegre del Castillo). *Podarcis galerai* sp. nov. occupies a range of arid Mediterranean landscapes characterized by a strong human impact and a poor xerophytic and thermophilous vegetation. A vast proportion of the distribution range is a high plateau area characterized by a remarkable aridity and semi-desertic conditions, where lizards are located in rocky outcrops on vacant lands (Puebla de Don Fadrique), in ruins of old buildings, artificial rock fences or in breakwaters of reservoirs (Embalse de los Rodeos and Embalse de la Pedrera).

Discussion

The mtDNA gene trees obtained show a clearer phylogeographical pattern than in previous attempts to unravel the history of the species complex (see, for example, Kaliontzopoulou et al., 2011). The most basal node in the tree gives rise to four groups: a monophyletic group including *P. muralis*, a western group (*P. carbonelli*, *P. virescens*, *P. guadarramae* and *P. bocagei*), a south-eastern group (*P. vaucheri*, Valencia, *P. h. sensu stricto* (Albacete/Murcia) and *P. galerai* sp. nov. (Galera)) and a low supported north-eastern group represented by *P. liolepis* (Figure 2). This low support could be due to the lack of genetic data from the entire distribution range of *P. liolepis*, as corroborated by the high genetic diversity showed on TCS networks for nuclear genes. An interesting result was that *P. liolepis* was not found to be the sister group of *P. galerai* sp. nov., in contrast to what has been indicated in other studies, which suggests that *P. liolepis* is a sister lineage to all remaining lineages of Iberian *Podarcis* (Pinho et al., 2006; Kaliontzopoulou et al., 2011). The species delimitation and clustering analysis based on mtDNA identified three groups in the SE region (Valencia lineage, *P. h. sensu stricto* and *P. galerai* sp. nov.). Even though species delimitation analysis based on nuclear loci and combined dataset (mtDNA+nuclear) provided support for treatment of the Valencia lineage, *P. h. sensu stricto* and *P. galerai* sp. nov. as different species, the species tree output in BPP and STACEY analyses could only provide significant support for the early divergence of *P. vaucheri*, unlike the findings based on mtDNA. All the species delimitation analysis based on both mtDNA and nuclear loci reported the same number of independent evolutionary units, therefore no gene flow or introgression was detected, contrary to the different number of clusters found using only mtDNA data, or morphological and nuclear data between *P. hispanicus* lineages in Renoult et al. (2009).

Networks based on nuclear genes indicated the uniqueness of *P. muralis* and *P. vaucheri* but showed a common haplotype between geographically close groups such as *P. virescens*, *P. liolepis*, *P. galerai* sp. nov., *P. h. sensu stricto* or the Valencia lineage. A posterior differentiation of the defined groups is also represented with the presence of species-specific haplotypes.

The SE region of the Iberian Peninsula presents a complex geological history, highlighting the connection between the Mediterranean Sea and the Atlantic Ocean through the Betic corridor during the middle Miocene (Serravallian/Tortonian; 12.8-7.2 Ma) and the definitive closure during the Messinian (7.2-5.33 Ma) triggering the start of the Messinian Salinity Crisis (5.97-5.33 Ma) connecting Africa and the Iberian Peninsula (Krijgsman et al., 1996, 2000, 2018). The geological instability present during these periods is thought to have caused the origin of many groups of organisms (Carranza et al., 2004b; Busack et al., 2005; Pinho et al., 2006; Kaliontzopoulou et al., 2011). We found that *P. galerai* sp. nov. split from the SE Iberian *Podarcis* group (Valencia lineage, *P. h. sensu stricto* and *P. vaucheri*) at 12.48 Ma. During this period, the Betic corridor was a connection between the Mediterranean Sea and the Atlantic Ocean dividing the Iberian Peninsula at the Betic cordillera. This scenario could have caused the isolation of *P. galerai* sp. nov. distributed in the south of this region. The separation between *P. liolepis* (distributed in the north-east) and the large clade of western lineages, including *P. carbonelli*, *P. virescens*, *P. guadarramae* and *P. bocagei*, also occurred during this period (12.58 Ma). The divergence between the Valencia lineage and the nominotypical taxon *P. h. sensu stricto* occurred at 7.11 Ma. This period corresponds with the end of the Tortonian, when the Betic Corridor became colonizable by land with the definitive closure of the Betic Strait, causing the connection between the north and south regions of the Iberian Peninsula and Africa.

According to our genetic results, lizards from Laderas del Campillo (Murcia province) belong to the Albacete/Murcia lineage. This is the closest point to the restricted type locality of Monteagudo (Geniez et al., 2007) where we found *Podarcis* lizards. Even though *P. galerai* sp. nov. samples present morphologically similar traits, samples from the Albacete/Murcia lineage were identified as the nominal form of the complex, as this lineage shares almost all morphological features proposed by Geniez et al. (2007) for the name-bearing type specimens.

In addition, our findings support a clear delimitation of *P. galerai* sp. nov., on one hand, and the group that encompasses the lineages from Valencia, *P. h. sensu stricto* and North Africa, on the other. This separation is noteworthy and supports full species status for the Galera lineage. The systematic status of the Valencia lineage remains unclear with additional genetic information on the lineage, wider sampling of its geographical range and a full morphological comparison with *P. h. sensu stricto* and *P. galerai* sp. nov. required before full species status can be assigned.

It is difficult to stablish the sympatry or non-sympatry of different lineages/species of *Podarcis* on the Iberian Peninsula. Previous studies evidenced a parapatric distribution in the easterns Iberian Peninsula; in contrast, in the west, species like *P. carbonelli* and *P. guadarramae* are largely sympatric in their distribution ranges, as well as *P. bocagei* and *P. guadarramae*. Further strict boundaries were proposed to separate eastern and southern species, such as *P. vaucheri*, *P. liolepis*, *P. virescens* and *P. hispanicus*. The limits among these species of the complex are not yet well defined due to samples being required from the entire distribution area. It is likely that the distribution areas will overlap, as it is the norm in Western Iberia and in other areas of the Mediterranean basin.

The only way to take into account the extraordinary diversity of mitochondrial lineages of Iberian *Podarcis* is to document their morphological characteristics and make taxonomic decisions. We agree with the important argument that only formal naming of different lineages will allow these new species to be considered in conservation policies (Geniez et al., 2007). We can always expect new discoveries or deeper analyses to change the general picture in the near future and, perhaps, certain taxa will change status, such as the Valencia lineage.

Despite both phylogenetic and species delimitation analyses corroborated with multiple methodological approaches and using different datasets (mtDNA, nuclear loci or combined data) showed high supports for the different SE *Podarcis* groups, genome-wide approaches would help to confirm these phylogenetic relationships and adress the issues of introgression. Furthermore, to solve the puzzle of lineages of this species complex would be necessary to obtain a better picture of the distribution of *Podarcis* genus in the Iberian Peninsula. It could be particularly important to study the *P. liolepis* and Valencia lineages in a deeper way, including a greater number of individuals and locations. In addition, a global morphological study of *P. hispanicus* complex would be of interest and would help to clarify the wide diversity presents in this species complex.

In conclusion, this study provides a large genetic dataset of mtDNA and nuclear sequences of the *P. hispanicus* complex based on a greater number of individuals and locations than in previous studies. These results allow a deeper phylogenetic analysis on SE *Podarcis* lineages that contributes to elucidating the controversy regarding this region. Our phylogenetic tree shows the group including the Valencia lineage and *P. h. sensu stricto*, as sister group of the North African form (*P. vaucheri*). *Podarcis galerai* sp. nov. form a monophyletic group, separated (~12 Ma) from the rest of the SE and North African forms, in contrast to previous studies based on a very low number of individuals that showed the closest phylogenetic relationship between *P. galerai* sp. nov. and *P. liolepis* (Pinho et al., 2006; Kaliontzopoulou et al., 2011). Our results support the elevation of *P. galerai* sp. nov. to full species rank.

Supplementary Table 1. Identification number, species, code number, geographic location and GenBank Accession Numbers for all the samples used in this study. Codes in bold represent the sequences generated in this study.

	Species	Code	Country	Locality	Lat	Long	12S	CR	CYTB	NADH	RAG1	MC1R
1	*P. vaucheri*	Vau01	Spain	Chafarinas Islands	35.182	-2.430	KT030729.1	KT030727.1	KT030723.1	KT030725.1	**MN651838**	**MN651708**
2	*P. vaucheri*	Vau02	Morocco	Oukaïmeden	31.202	-7.858	KT030730.1	KT030728.1	KT030724.1	KT030726.1		**MN651709**
3	*P. carbonelli*	CAR01	Portugal	Lagoa Comprida	40.365	-7.647	KT030681.1	KT030679.1	KT030675.1	KT030677.1	**MN651839**	**MN651710**
4	*P. bocagei*	BN3	Spain	La Baña	42.289	-6.670	**MN649217**	**MN651572**	**MN651598**	**MN651659**	**MN651840**	**MN651711**
5	*P. bocagei*	BN5	Spain	La Baña	42.289	-6.670	**MN649217**	**MN651572**	**MN651598**	**MN651660**	**MN651841**	**MN651712**
6	*P. g. lusitanicus*	CAR02	Portugal	Lagoa Comprida	40.365	-7.647	KT030682.1	KT030680.1	KT030676.1	KT030678.1	**MN651842**	**MN651713**
7	*P. g. lusitanicus*	HN1	Spain	La Baña	42.289	-6.670	**MN649218**	**MN651573**	**MN651599**	**MN651661**	**MN651843**	**MN651714**
8	*P. g. guadarramae*	HE4	Spain	Escorial de la Sierra, Salamanca	40.614	-5.951	**MN649219**	**MN651574**	**MN651600**	**MN651662**	**MN651844**	**MN651715**
9	*P. g. guadarramae*	HM3	Spain	Mogarraz, Salamanca	40.501	-6.063	KT030705.1	**MN651575**	**MN651601**	**MN651663**	**MN651845**	**MN651716**
10	*P. g. guadarramae*	HS1	Spain	Los Santos, Salamanca	40.545	-5.797	**MN649220**	KT030700.1	**MN651602**	**MN651664**	**MN651846**	**MN651717**
11	*P. g. guadarramae*	HC1	Spain	Cabrerizos, Salamanca	40.979	-5.608	KT030705.1	KT030700.1	KT030688.1	KT030694.1	**MN651847**	**MN651718**
12	*P. g. guadarramae*	HT1	Spain	Tiermes, Soria	41.336	-3.153	**MN649221**	**MN651576**	**MN651603**	**MN651665**	**MN651848**	**MN651719**
13	*P. g. guadarramae*	HP2	Spain	Pedro, Soria	41.317	-3.194	**MN649221**	**MN651576**	**MN651603**	**MN651665**	**MN651849**	**MN651720**
14	*P. g. guadarramae*	His3	Spain	Escorial de la sierra, Salamanca	40.614	-5.951	**MN649219**	**MN651574**	**MN651600**	**MN651662**	**MN651850**	**MN651721**
15	*P. g. guadarramae*	HGF1	Spain	Fuenfría, Madrid	40.741	-4.066	**MN649221**	**MN651574**	**MN651604**	**MN651665**	**MN651851**	**MN651722**
16	*P. g. guadarramae*	HGF2	Spain	Fuenfría, Madrid	40.741	-4.066	**MN649221**	**MN651576**	**MN651605**	**MN651665**	**MN651852**	**MN651723**
17	*P. g. guadarramae*	HGF5	Spain	Fuenfría, Madrid	40.741	-4.066	**MN649221**	**MN651576**	**MN651606**	**MN651666**	**MN651853**	**MN651724**
18	*P. g. guadarramae*	HGF	Spain	Fuenfría, Madrid	40.741	-4.066	**MN649221**	**MN651577**	**MN651605**	**MN651665**	**MN651854**	**MN651725**
19	*P. g. guadarramae*	HGG1	Spain	Navacerrada, Madrid	40.722	-4.024	**MN649221**	**MN651576**	**MN651607**	**MN651665**	**MN651855**	**MN651726**
20	*P. g. guadarramae*	HGG2	Spain	Navacerrada, Madrid	40.722	-4.024	**MN649221**	**MN651576**	**MN651608**	**MN651667**	**MN651856**	**MN651727**
21	*P. g. guadarramae*	HGG3	Spain	Navacerrada, Madrid	40.722	-4.024	**MN649221**	**MN651577**	**MN651605**	**MN651665**	**MN651857**	**MN651728**
22	*P. g. guadarramae*	HGG4	Spain	Navacerrada, Madrid	40.722	-4.024	**MN649221**	**MN651576**	**MN651605**	**MN651668**	**MN651858**	**MN651729**
23	*P. g. guadarramae*	HGG5	Spain	Navacerrada, Madrid	40.722	-4.024	**MN649221**	**MN651576**	**MN651609**	**MN651669**	**MN651859**	**MN651730**
24	*P. virescens*	HD1	Spain	Don Benito, Badajoz	38.956	-5.864	KT030704.1	KT030699.1	KT030687.1	KT060693.1	**MN651860**	**MN651731**
25	*P. virescens*	HVA1	Spain	Aranjuez, Madrid	39.995	-3.672	**MN649222**	**MN651578**	**MN651610**	**MN651670**	**MN651861**	**MN651732**
26	*P. virescens*	HVA2	Spain	Aranjuez, Madrid	39.995	-3.672	**MN649223**	**MN651578**	**MN651610**	**MN651670**	**MN651862**	**MN651733**
27	*P. virescens*	HVA3	Spain	Aranjuez, Madrid	39.995	-3.672	**MN649223**	**MN651578**	**MN651610**	**MN651670**	**MN651863**	**MN651734**
28	*P. virescens*	HVA4	Spain	Aranjuez, Madrid	39.995	-3.672	**MN649223**	**MN651578**	**MN651610**	**MN651670**	**MN651864**	**MN651735**

29	*P. virescens*	HVP2	Spain	Pedrezuela, Madrid	40.750	-3.612	**MN649224**	**MN651578**	**MN651611**	**MN651671**	**MN651865**	**MN651736**
30	*P. virescens*	HVP3	Spain	Pedrezuela, Madrid	40.750	-3.612	**MN649224**	**MN651578**	**MN651612**	**MN651670**	**MN651866**	**MN651737**
31	*P. virescens*	HVP4	Spain	Pedrezuela, Madrid	40.750	-3.612	**MN649224**	**MN651578**	**MN651612**	**MN651670**	**MN651867**	**MN651738**
32	*P. virescens*	HVP5	Spain	Pedrezuela, Madrid	40.750	-3.612	**MN649224**	**MN651578**	**MN651612**	**MN651670**	**MN651868**	**MN651739**
33	Galera lineage	Gale1	Spain	Galera, Granada	37.739	-2.563	**MN649225**	**MN651579**	**MN651613**	**MN651672**	**MN651869**	**MN651740**
34	Galera lineage	Gale3	Spain	Galera, Granada	37.739	-2.563	**MN649225**	**MN651579**	**MN651613**	**MN651672**	**MN651870**	**MN651741**
35	Galera lineage	Gale5	Spain	Galera, Granada	37.739	-2.563	**MN649225**	**MN651579**	**MN651613**	**MN651672**	**MN651871**	**MN651742**
36	Galera lineage	Df1	Spain	Puebla de Don Fadrique, Granada	37.911	-2.398	**MN649225**	**MN651580**	**MN651613**	**MN651672**	**MN651872**	**MN651743**
37	Galera lineage	Df3	Spain	Puebla de Don Fadrique, Granada	37.911	-2.398	**MN649225**	**MN651581**	**MN651613**	**MN651672**		**MN651744**
38	Galera lineage	Ph16	Spain	Orihuela, Alicante	38.085	-0.948	**MN649225**	**MN651580**	**MN651614**	**MN651673**	**MN651873**	**MN651745**
39	Galera lineage	Ph21	Spain	Embalse de la Pedrera, Orihuela	38.032	-0.870	**MN649225**	**MN651580**	**MN651615**	**MN651674**		**MN651746**
40	Galera lineage	Ph22	Spain	Embalse de la Pedrera, Orihuela	38.032	-0.870	**MN649226**	**MN651580**	**MN651614**	**MN651673**		**MN651747**
41	Galera lineage	Ro1	Spain	Embalse de los Rodeos, Murcia	38.043	-1.295	**MN649225**	**MN651580**	**MN651616**	**MN651673**	**MN651874**	**MN651748**
42	Galera lineage	Ro2	Spain	Embalse de los Rodeos, Murcia	38.043	-1.295	**MN649225**	**MN651580**	**MN651617**	**MN651673**		**MN651749**
43	Galera lineage	AB8	Spain	Las Canteras, Murcia	37.634	-1.041	**MN649225**	**MN651580**	**MN651618**	**MN651675**	**MN651875**	**MN651750**
44	Galera lineage	AB9	Spain	Las Canteras, Murcia	37.634	-1.041	**MN649225**	**MN651580**	**MN651614**	**MN651676**	**MN651876**	**MN651751**
45	Valencia lineage	HB2	Spain	Burjassot, Valencia	39.511	-0.414	KT030701.1	KT030695.1	**MN651619**	KT030689.1	**MN651877**	**MN651752**
46	Valencia lineage	PHP3	Spain	Puebla Salvador, Cuenca	39.563	-1.675	**MN649227**	**MN651582**	**MN651620**	**MN651677**	**MN651878**	
47	Valencia lineage	PHP4	Spain	Puebla Salvador, Cuenca	39.563	-1.675	**MN649227**	**MN651582**	**MN651621**	**MN651678**	**MN651879**	**MN651753**
48	Valencia lineage	PHCE2	Spain	Ciudad Encantada, Cuenca	40.208	-2.011	KT030701.1	**MN651582**	**MN651622**	KT030689.1	**MN651880**	**MN651754**
49	Valencia lineage	PHCE4	Spain	Ciudad Encantada, Cuenca	40.208	-2.011	KT030701.1	**MN651582**	**MN651623**	**MN651679**	**MN651881**	**MN651755**
50	bacete/Murcia lineage	Alb1	Spain	Sierra de la Oliva, Albacete	38.765	-0.971	**MN649228**	**MN651583**	**MN651624**	**MN651680**		**MN651756**
51	bacete/Murcia lineage	Alb2	Spain	Sierra de la Oliva, Albacete	38.765	-0.971	**MN649228**	**MN651583**	**MN651624**	**MN651680**		**MN651757**
52	bacete/Murcia lineage	Alb3	Spain	Montealegre del Castillo, Albacete	38.826	-1.341	**MN649228**	**MN651583**	**MN651625**	**MN651680**	**MN651882**	**MN651758**
53	bacete/Murcia lineage	Alb4	Spain	Montealegre del Castillo, Albacete	38.826	-1.341	**MN649228**	**MN651584**	**MN651626**	**MN651680**	**MN651883**	**MN651759**
54	bacete/Murcia lineage	Alb6	Spain	Cañada del Provencio, Albacete	38.518	-2.353	**MN649228**	**MN651583**	**MN651627**	**MN651680**	**MN651884**	**MN651760**
55	bacete/Murcia lineage	Seg2	Spain	Sierra Callosa del Segura, Alicante	38.123	-0.895	**MN649228**	**MN651583**	**MN651628**	**MN651680**	**MN651885**	**MN651761**
56	bacete/Murcia lineage	Cmp1	Spain	Laderas del Campillo, Murcia	38.060	-1.092	**MN649228**	**MN651583**	**MN651629**	**MN651681**	**MN651886**	**MN651762**
57	bacete/Murcia lineage	Cmp2	Spain	Laderas del Campillo, Murcia	38.060	-1.092	**MN649228**	**MN651583**			**MN651887**	**MN651763**
58	bacete/Murcia lineage	Cmp4	Spain	Laderas del Campillo, Murcia	38.060	-1.092	**MN649228**	**MN651583**	**MN651629**	**MN651681**		**MN651764**
59	*P. liolepis*	AB0	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	**MN651585**	**MN651630**	**MN651682**	**MN651888**	**MN651765**

60	*P. liolepis*	AB1	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	**MN651585**	**MN651631**	**MN651683**	**MN651889**	**MN651766**
61	*P. liolepis*	AB2	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	**MN651585**	**MN651632**	**MN651683**	**MN651890**	**MN651767**
62	*P. liolepis*	AB5	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	**MN651585**	**MN651632**	**MN651685**	**MN651893**	**MN651770**
63	*P. liolepis*	AB7	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	**MN651585**	**MN651633**	**MN651684**	**MN651894**	**MN651771**
64	*P. liolepis*	AF1	Spain	Foradada, Columbretes Islands	39.875	0.671	KT030702.1	**MN651585**	**MN651636**		**MN651908**	**MN651785**
65	*P. liolepis*	AF2	Spain	Foradada, Columbretes Islands	39.875	0.671	KT030702.1	**MN651585**	**MN651636**	**MN651682**	**MN651909**	**MN651786**
66	*P. liolepis*	AO2	Spain	Lobo, Columbretes Islands	39.875	0.672	KT030702.1	**MN651585**	**MN651636**		**MN651910**	**MN651787**
67	*P. liolepis*	AO3	Spain	Lobo, Columbretes Islands	39.875	0.672	KT030702.1	**MN651585**	**MN651636**	**MN651682**	**MN651911**	**MN651788**
68	*P. liolepis*	AM1	Spain	Mancolibre, Columbretes Islands	39.895	0.690	KT030702.1	**MN651585**	**MN651632**		**MN651912**	**MN651789**
69	*P. liolepis*	AM2	Spain	Mancolibre, Columbretes Islands	39.895	0.690	KT030702.1	**MN651585**	**MN651632**	**MN651686**	**MN651913**	**MN651790**
70	*P. liolepis*	HL1	Spain	Sazajarra, Rioja	42.588	-2.961	KT030703.1	KT030698.1	**MN651637**	KT030692.1	**MN651914**	**MN651791**
71	*P. liolepis*	PL3	France	Pêch de Foix	42.963	1.623	**MN649229**	**MN651586**	**MN651638**	**MN651687**	**MN651915**	**MN651792**
72	*P. liolepis*	PL60	France	Vaychis	42.748	1.840	KT030702.1	**MN651587**	**MN651639**	**MN651682**	**MN651916**	**MN651793**
73	*P. liolepis*	HF9	Spain	Peñagolosa, Castellon	40.245	-0.376	KT030702.1	KT030697.1	KT030685.1	KT030691.1	**MN651917**	**MN651794**
74	*P. liolepis*	HJ2	Spain	Peñagolosa, Castellon	40.245	-0.376	KT030702.1	**MN651587**	KT030684.1	KT030690.1	**MN651918**	**MN651795**
75	*P. liolepis*	HJ3	Spain	Peñagolosa, Castellon	40.245	-0.376	KT030702.1	**MN651587**	**MN651640**	**MN651688**	**MN651919**	**MN651796**
76	*P. liolepis*	HJ4	Spain	Peñagolosa, Castellon	40.245	-0.376	KT030702.1	**MN651587**	**MN651641**	**MN651689**	**MN651920**	**MN651797**
77	*P. liolepis*	HPC8	Spain	Peñagolosa, Castellon	40.245	-0.376	KT030702.1	**MN651587**	**MN651640**	**MN651690**	**MN651921**	**MN651798**
78	*P. liolepis*	MPC5	Spain	Peñagolosa, Castellon	40.245	-0.376	KT030702.1	**MN651587**	KT030707.1	KT030709.1	**MN651922**	**MN651799**
79	*P. liolepis*	MPC6	Spain	Peñagolosa, Castellon	40.245	-0.376	KT030702.1	**MN651588**	**MN651642**	**MN651690**	**MN651923**	**MN651800**
80	*P. liolepis*	HP118	Spain	Peñagolosa, Castellon	40.245	-0.376	KT030702.1	**MN651587**	**MN651643**	**MN651691**	**MN651924**	**MN651801**
81	*P. liolepis*	HP218	Spain	Peñagolosa, Castellon	40.245	-0.376	KT030702.1	**MN651587**	**MN651640**	**MN651692**	**MN651925**	**MN651802**
82	*P. liolepis*	HP3	Spain	Peñagolosa, Castellon	40.245	-0.376	**MN649230**	**MN651587**	**MN651644**	**MN651693**	**MN651926**	**MN651803**
83	*P. liolepis*	HP4	Spain	Peñagolosa, Castellon	40.245	-0.376	KT030702.1	**MN651587**	**MN651645**	**MN651694**	**MN651927**	**MN651804**
84	*P. liolepis*	HP5	Spain	Peñagolosa, Castellon	40.245	-0.376	KT030702.1	**MN651589**	**MN651646**	KT030690.1	**MN651928**	**MN651805**
85	*P. liolepis*	HP8	Spain	Peñagolosa, Castellon	40.245	-0.376	KT030702.1	**MN651585**	**MN651642**	**MN651690**	**MN651929**	**MN651806**
86	*P. liolepis*	HP9	Spain	Peñagolosa, Castellon	40.245	-0.376	KT030702.1	**MN651587**	**MN651647**	**MN651690**	**MN651930**	**MN651807**
87	*P. liolepis*	HP10	Spain	Peñagolosa, Castellon	40.245	-0.376	KT030702.1	**MN651587**	**MN651641**	KT030709.1	**MN651931**	**MN651808**
88	*P. liolepis*	HP11	Spain	Peñagolosa, Castellon	40.245	-0.376	KT030702.1	**MN651590**	**MN651648**	KT030690.1	**MN651932**	**MN651809**
89	*P. muralis*	Mpfn7	Spain	Peñagolosa, Castellon	40.245	-0.376	**MN649231**	**MN651591**	**MN651649**	**MN651695**	**MN651933**	**MN651810**
90	*P. muralis*	MGV1	Spain	Villaviciosa, Asturias	43.482	-5.433	**MN649232**	**MN651592**	**MN651650**	**MN651696**	**MN651934**	**MN651811**

91	*P. muralis*	MLL1	Spain	Villaviciosa, Asturias	43.482	-5.433	**MN649232**	**MN651592**	**MN651650**	**MN651697**	**MN651935**	**MN651812**
92	*P. muralis*	MPN1	Spain	Pendueles, Asturias	43.397	-4.633	**MN649232**	**MN651593**	**MN651651**	**MN651699**	**MN651938**	**MN651815**
93	*P. muralis*	MPG4	Spain	Pto. San Glorio, Cantabria	43.458	-3.814	**MN649232**	**MN651593**	**MN651653**	**MN651699**	**MN651941**	**MN651818**
94	*P. muralis*	MVB1	Spain	San Vicente de la Barquera, Cantabria	43.381	-4.399	**MN649232**	**MN651593**	**MN651651**	**MN651700**	**MN651943**	**MN651820**
95	*P. muralis*	MSP1	Spain	Cervera, Castilla y León	42.867	-4.500	**MN649232**	**MN651593**	**MN651651**	**MN651701**	**MN651944**	**MN651821**
96	*P. muralis*	MSP2	Spain	Cervera, Castilla y León	42.867	-4.500	MN649232	MN651593	MN651652	MN651699	MN651945	MN651822
97	*P. muralis*	MLP1	Spain	La Pinilla, Castilla y León	41.222	-3.479	KT030712.1	KT030710.1	KT030706.1	KT030708.1	**MN651949**	**MN651826**
98	*P. muralis*	MSS1	Spain	Selorio, Asturias	43.515	-5.362	**MN649232**	**MN651593**	**MN651651**	**MN651703**	**MN651950**	**MN651827**
99	*P. muralis*	MP2	France	Moulis	42.960	1.092	**MN649233**	**MN651594**	**MN651654**	**MN651704**	**MN651951**	**MN651828**
100	*P. muralis*	MP3	France	Moulis	42.960	1.092	**MN649234**	**MN651595**	**MN651655**	**MN651705**	**MN651952**	**MN651829**
101	*P. muralis*	MP7	France	Moulis	42.960	1.092	**MN649235**	**MN651596**	**MN651656**	**MN651704**	**MN651955**	**MN651832**
102	*P. muralis*	MP11	France	Moulis	42.960	1.092	**MN649235**	**MN651594**	**MN651656**	**MN651704**	**MN651958**	**MN651835**
103	*P. muralis*	MP13	France	Vaychis	42.748	1.840	**MN649234**	**MN651595**	**MN651658**	**MN651705**	**MN651959**	**MN651836**
104	*P. muralis*	MP14	France	Vaychis	42.748	1.840	**MN649235**	**MN651597**	**MN651656**	**MN651707**	**MN651960**	**MN651837**
	Outgroup											
	P. lilfordi	D22	Spain	Dragonera, Balearic Islands	39.584	2.320	EF694761.1	EF694773.1	KC623944.1	EU006730.1	MN651964	JX126631.1
	P. lilfordi	Cr1	Spain	Es Colomer, Balearic Islands	39.945	3.131	EF694760.1	EF694775.1	EF694801.1/ EF990524.1	EU006737.1		
	P. lilfordi	Cp1	Spain	Cabrera harbour, Balearic Islands	39.151	2.934	EF694764.1	EF694780.1	KF003361.1/EF990531.1	EU006743.1	MN651963	JX126652.1
	P. lilfordi	Cf	Spain	Cabrera lighthouse, Balearic Islands	39.129	2.921					MN651961	JX126649.1
	P. lilfordi	X3	Spain	Estel Xapat, Balearic Islands	39.125	2.939	EF694765.1	EF694785.1	EF694807.1/ EF990539.1	EU006753.1		
	P. lilfordi	AG1	Spain	Addaia Gran, Balearic Islands	40.016	4.210	EF694766.1	EF694786.1	EF694809.1/ EF694826.1	EU006756.1		
	P. lilfordi	CI	Spain	Binicodrell, Balearic Islands	39.916	4.030					MN651962	JX126640.1
	P. pityusensis	Alg1	Spain	Alga, Balearic Islands	38.977	1.532	EF694768.1	EF694794.1	JX852075.1	JX852119.1		
	P. pityusensis	Eiv1	Spain	Ibiza, Balearic Islands	38.907	1.421	EF694768.1	EF694794.1	JX852050.1	JX852121.1	MN651965	JX126670.1
	P. pityusensis	Fort4	Spain	Formentera, Balearic Islands	38.753	1.436	EF694768.1	EF694794.1	JX852075.1	JX852121.1	MN651966	JX126678.1
	P. pityusensis	Fx	Spain	Formentera, Balearic Islands	38.706	1.429					MN651967	JX126678.1

Supplementary Table 2. Morphometric and scalation characters of adult males and females of the Albacete/Murcia and Galera lineages. For each group and character, the average ± standard error, maximum and minimum values, and sample size are given. Abbreviations: SVL, snout-vent length; TL, intact tail length; PL, pileus length; HH, head height; HW, head width; HLL, left hind limb length; LAM, left fourth digit lamellae; FEM, femoralia; GUL, gularia; DOR, dorsalia; VENT, ventralia; COLL, collaria.

Lineage	SVL	TL	Weight	PL	HH	HW	HLL	LAM	FEM	GUL	DOR	VENT	COLL
Albacete/Murcia Adult males	46.21 ± 0.59 44.0-49.0 (8)	106.5 ± 1.5 105-108 (2)	2.45 ± 0.06 2.2-2.7 (8)	10.61± 0.2 9.85-11.27 (8)	4.13 ± 0.11 3.69-4.69 (8)	4.7 ± 0.07 4.47-5.02 (8)	22.59 ± 0.51 20.69-24.74 (8)	24.43 ± 0.57 22-26 (8)	17.75 ± 0.56 15-20 (8)	29.62 ± 0.71 27-33 (8)	63.75 ± 2.05 56-73 (8)	21.75 ± 0.25 21-23 (8)	9.88 ± 0.23 9-11 (8)
Albacete/Murcia Adult females	43.62 ± 0.38 43.0-44.5 (4)	78 (1)	1.68 ± 0.12 1.5-2 (4)	9.27 ± 0.07 9.13-9.34 (3)	3.63± 0.14 3.29-3.93 (4)	3.89 ± 0.14 3.5-4.08 (4)	18.38 ± 0.31 17.67-18.98 (4)	23 ± 0.58 22-24 (3)	15.25 ± 0.63 14-17 (4)	27.75 ± 0.75 26-29 (4)	68.25 ± 2.25 62-72 (4)	25 ± 0.58 24-26 (4)	9.5 ± 0.29 9-10 (4)
Galera Adult males	47.69 ± 0.71 42.0-52.5 (16)	102.57±3.48 87-115 (7)	2.62 ± 0.16 2.0-3.8 (15)	11.01 ± 0.2 9.42-12.38 (16)	4.82± 0.35 4.09-9,95 (16)	5.09 ± 0.08 4.63-5.63 (16)	24.04 ± 0.52 21.25-28.26 (16)	24 ± 0.35 22-27 (16)	15.25 ± 0.34 13-19 (16)	25.5 ± 0.44 23-29 (16)	64.93 ± 1.65 56-78 (15)	23.44 ± 0.4 21-26 (16)	9.75 ± 0.21 8-11 (16)
Galera Adult females	44.5 ± 1.36 38.5-49 (9)	90.82 ± 2.18 81.95 (6)	2.04 ± 0.12 1.5-2.5 (8)	9.36 ± 0.21 8.48-10.4 (9)	3.93 ± 0.14 3.36-4.44 (9)	4.34 ± 0.2 3.29-5.03 (9)	20.02 ± 0.57 17.22-21.89 (8)	24.17 ± 0.6 22-26 (6)	16 ± 0.63 13-19 (8)	26.75 ± 0.62 25-29 (8)	63.71 ± 2 55-73 (7)	26.57 ± 0.3 25-27 (7)	10.14 ± 0.4 9-12 (7)

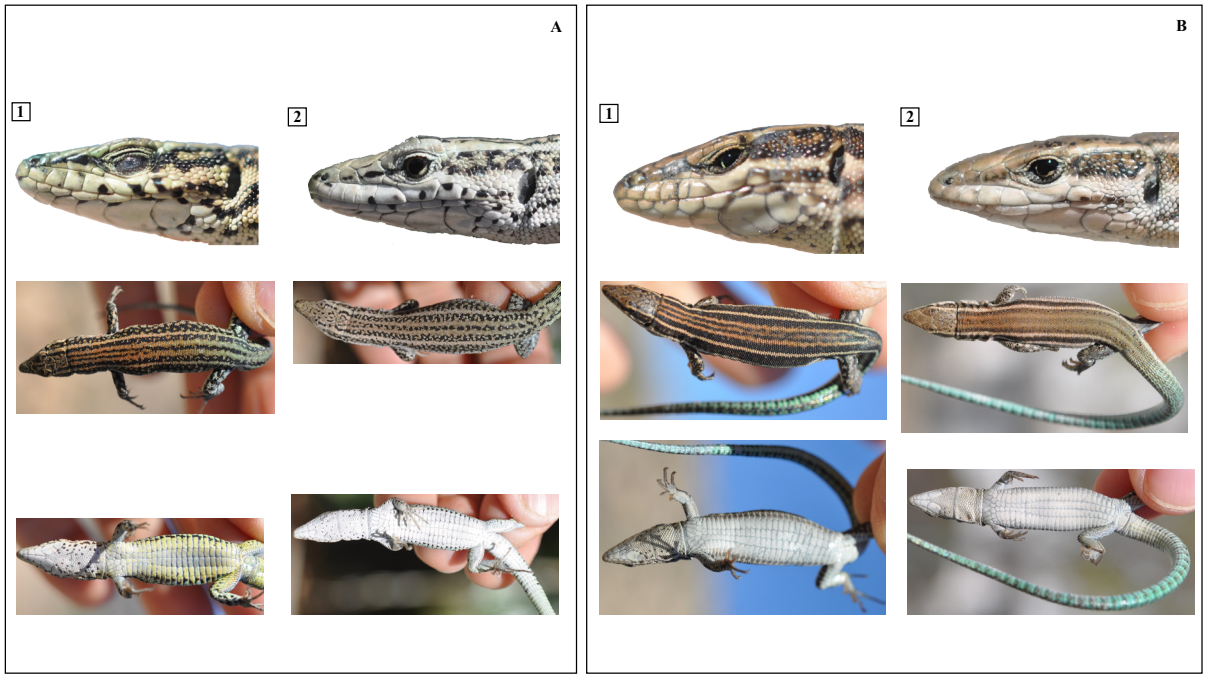
Supplementary Table 3. Comparison between the Albacete/Murcia lineage, Galera lineage and the nominotypical taxon described by Geniez et al. (2007).

Descriptive traits	Geniez et al. (2007)	Albacete/Murcia lineage	Galera lineage
Continuation of light dorsolateral stripes and dark supra-dorsolateral stripes along parietal plates	96% of lizards	92% of lizards	87.5% of lizards
Lack of masseteric plate in both sides	98.5%	100%	100%
Small body size	SVL<50 mm in 99.5% Max=51.5 mm	Average SVL= 46.21 mm Max=49 mm	Average SVL= 47.69 mm Max= 52.5 mm
Pointed snout and flattened head	HH/PL< 0.46 in 98.5 % of lizards	Average HH/PL= 0.39 ± 0.01 range: 0.35-0.42	Average HH/PL = 0.43 ± 002 range: 0.38-0.85
Presence of vertebral line	83%	83.3%	50%
Upper bifurcation of vertebral line	72.5%	83.3%	45.83%
Presence of dark spots in outer ventral scales	93%	92%	70.83%
Absence of green coloration on the dorsum	100%	100%	100%
Belly color	97% of lizards with white color	66.7% of lizards with white color, 33.3% with yellowish bellies	75% of lizards with white color, 15% with yellowish bellies and 10% with orange belly
Absence of blue spots on outer ventral scales	93%	75%	58.82%
Blue tail in juveniles	100%	100%	100%

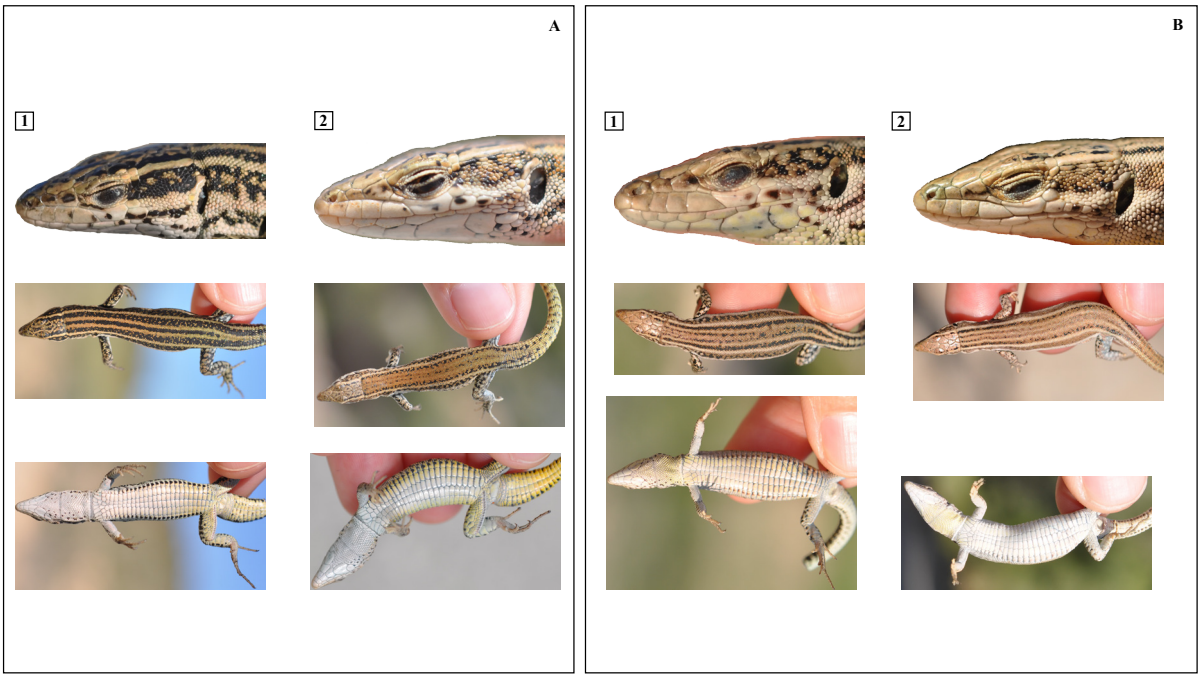
Supplementary Table 4. Colour codes in the two lineages under study, Galera (G) and Albacete/Murcia (A/M) (colour codes according to Köhler, 2012). For each body region, we provide the number of adult lizards where each particular colour was observed.

Colour	Background dorsum		Pileus		Flanks		Belly		Gular		ocelli		Dorsolateral stripes		Ventral tail		Upper tail	
	G	A/M	G	A/M	G	A/M	G	A/M	G	A/M	G	A/M	G	A/M	G	A/M	G	A/M
1. Pale Buff							2	8	4	6			1					
2. Light Buff							6	3	1				1		1			
3. Pale Pinkish Buff										2				1		3		
5. Buff															1			
6. Buff-Yellow							1											
11. Pale Horn Color							2		3						2	1		
12. Cream Color									1	1					1	2		
13. Light Yellow Ocher									1						1	1		
16. Olive Horn Color													1					5
17. Tawny Olive				1														1
18. Clay Color				1		1												
19. Drab	2	3	1	3		3												1
24. Antique Brown				1														
37. Verona Brown			1															
45. Dark Drab	1	1	1															
50. Cinnamon-Drab		1			1	2							2	3				
52. Cream White							1		1				1					
53. Straw Yellow															3			
58. Salmon Color						1												
97. Pale Greenish White																1		
99. Pale Green															1		1	
100. Light Yellow-Green																	2	
112. Pale Lime Green															1			
117. Olive Yellow																	1	
132. Parrot Green																	1	
141. Pale Emerald Green																	1	
142. Light Emerald Green															1			
143. Emerald Green																	1	
144. Light Paris Green																	1	
156. Cyan White											1							
157. Pale Cyan											1							
161. Robin's Egg Blue											1							
164. Light Medium Blue												1						
192. Sky Blue												1						
195. Lavender Blue											1							
196. Flaxflower Blue											1							
250. Light Flesh Color														1				
254. Beige					1				1				3	4				
255. Cinnamon	1	1	1														1	
256. Drab-Gray					3	1							2	1				
257. Medium Fawn Color													1					
258. Fawn Color						1												
260. True Cinnamon		1				1												

261. Smoky White							8		6	2					1			
262. Pearl Gray					2								2		3			
263. Light Smoke Gray															1			
265. Olive-Gray	2		2															1
266. Smoke Gray					2	1							4	1				
267. Smoke Gray	1	1				1												
268. Grayish Horn Color	1	1		1													1	
269. Light Drab	3			1													1	1
270. Ground Cinnamon	2	4	6	2	1	1												
271. Greenish Glaucous																	1	
272. Glaucous	1																	
274. Grayish Olive																	1	
276. Brownish Olive	3																	
277. Hair Brown					1													
278. Olive-Brown	4		3															
288. Light Bluish Gray															1			
296. Pale Neutral Gray					2								2					



Supplementary Figure 1. A-B. Morphology of Galera lineage. - A. Male specimens from Puebla de Don Fadrique (1) and Galera (2). - B. Female specimens from Puebla de Don Fadrique (1) and Galera (2).



Supplementary Figure 2. Morphology of Albacete/Murcia lineage. - A. Male specimens from Montealegre del Castillo (1) and Laderas del Campillo (2). - B. Female specimens from Caudete (1) and Callosa del Segura (2).

Additional information not included in the publication

Additional Table 1. Genetic diversity parameters and neutrality tests (Tajima's D and Fu and Li's F statistics) for mtDNA concatenated alignment (2,301 bp) from all the *Podarcis* popualtions from Iberian Peninsula and North African used in this study. n.s.: non-significant, (*): P<0.05, (**): P<0.02

Species	Number of sequences (N)	Number of segregating sites (S)	Number haplotypes (h)	Haplotype diversity (Hd)	Number of differences pairwise (K)	Nucleotide diversity (Pi)	Tajima's D (1989)	Fu and Li's F (1993)
P. vaucheri	2	66	2	1.000±0.500	66.000	0.0288±0.0144	-	-
P. bocagei	2	2	2	1.000±0.500	2.000	0.0009±0.0004	-	-
P.guadarramae	16	61	13	0.975±0.029	18.833	0.0082±0.0015	0.0339$^{n.s.}$	0.0479$^{n.s.}$
P. g. lusitanicus	2	24	2	1.000±0.500	24.000	0.0105±0.0052	-	-
P. virescens	9	66	5	0.833±0.098	15.556	0.0068±0.0044	-1.8436*	-2.1156**
P. galerai	12	14	10	0.955±0.057	3.712	0.0016±0.0003	-0.8502$^{n.s.}$	-0.8957$^{n.s.}$
P. hispanicus (Valencia)	5	13	5	1.000±0.126	5.600	0.0024±0.0005	-0.7449$^{n.s.}$	-0.7449$^{n.s.}$
P. hispanicus sensu stricto	8	12	6	0.929±0.084	3.821	0.0017±0.0003	-0.8770$^{n.s.}$	-0.8169$^{n.s.}$
P. liolepis (all)	47	123	27	0.895±0.040	12.006	0.0052±0.0014	-2.0880*	-2.5352*
P. liolepis (mainland)	19	113	19	1.000±0.017	19.754	0.0086±0.0028	-1.6637$^{n.s.}$	-1.4515$^{n.s.}$
P. liolepis (Columbretes)	28	10	8	0.698±0.087	1.907	0.0008±0.0002	-0.8291$^{n.s.}$	-0.0087$^{n.s.}$
P. muralis	32	91	19	0.948±0.021	29.365	0.0128±0.0009	1.0772$^{n.s.}$	1.0745$^{n.s.}$

Additional Table 2. Genetic diversity parameters and neutrality tests (Tajima's D and Fu and Li's F statistics) for RAG1 (939 bp) phased nuclear gene from all the *Podarcis* popualtions from Iberian Peninsula and North African used in this study. n.s.: non-significant, (*): P<0.05, (**): P<0.02

Species	Number of sequences (N)	Number of segregating sites (S)	Number haplotypes (h)	Haplotype diversity (Hd)	Number of differences pairwise (K)	Nucleotide diversity (Pi)	Tajima's D (1989)	Fu and Li's F (1993)
P. bocagei	8	8	6	0.893±0.111	3.250	0.0035±0.0006	0.2580$^{n.s.}$	0.2520$^{n.s.}$
P.g.guadarramae	32	9	9	0.815±0.050	1.484	0.0016±0.0002	-1.0292$^{n.s.}$	-0.7034$^{n.s.}$
P. g. lusitanicus	4	5	3	0.833±0.222	2.833	0.0030±0.0008	0.3719$^{n.s.}$	0.3514$^{n.s.}$
P.virescens	18	13	11	0.928±0.040	2.588	0.0028±0.0004	-1.1757$^{n.s.}$	-1.3038$^{n.s.}$
P. galerai	18	13	12	0.935±0.041	3.157	0.0034±0.0005	-0.6145$^{n.s.}$	-0.5073$^{n.s.}$
P. hispanicus (Valencia)	14	10	10	0.945±0.045	3.099	0.0033±0.0004	-0.4103$^{n.s.}$	0.1095$^{n.s.}$
P. hispanicus sensu stricto	12	4	3	0.591±0.108	1.030	0.0011±0.0005	-0.7813$^{n.s.}$	-1.3100$^{n.s.}$
P. liolepis (all))	102	46	36	0.890±0.024	2.997	0.0032±0.0002	-2.111*	-3.5275**
P. liolepis (mainland))	38	34	26	0.972±0.014	3.895	0.0042±0.0005	-1.8176*	-2.5525*
P. liolepis (Columbretes)	64	15	12	0.750±0.050	1.990	0.0021±0.0002	-1.0995$^{n.s.}$	-1.1286$^{n..s.}$
P.muralis	56	6	7	0.266±0.078	0.348	0.0004±0.0001	-1.8260*	-2.5549*

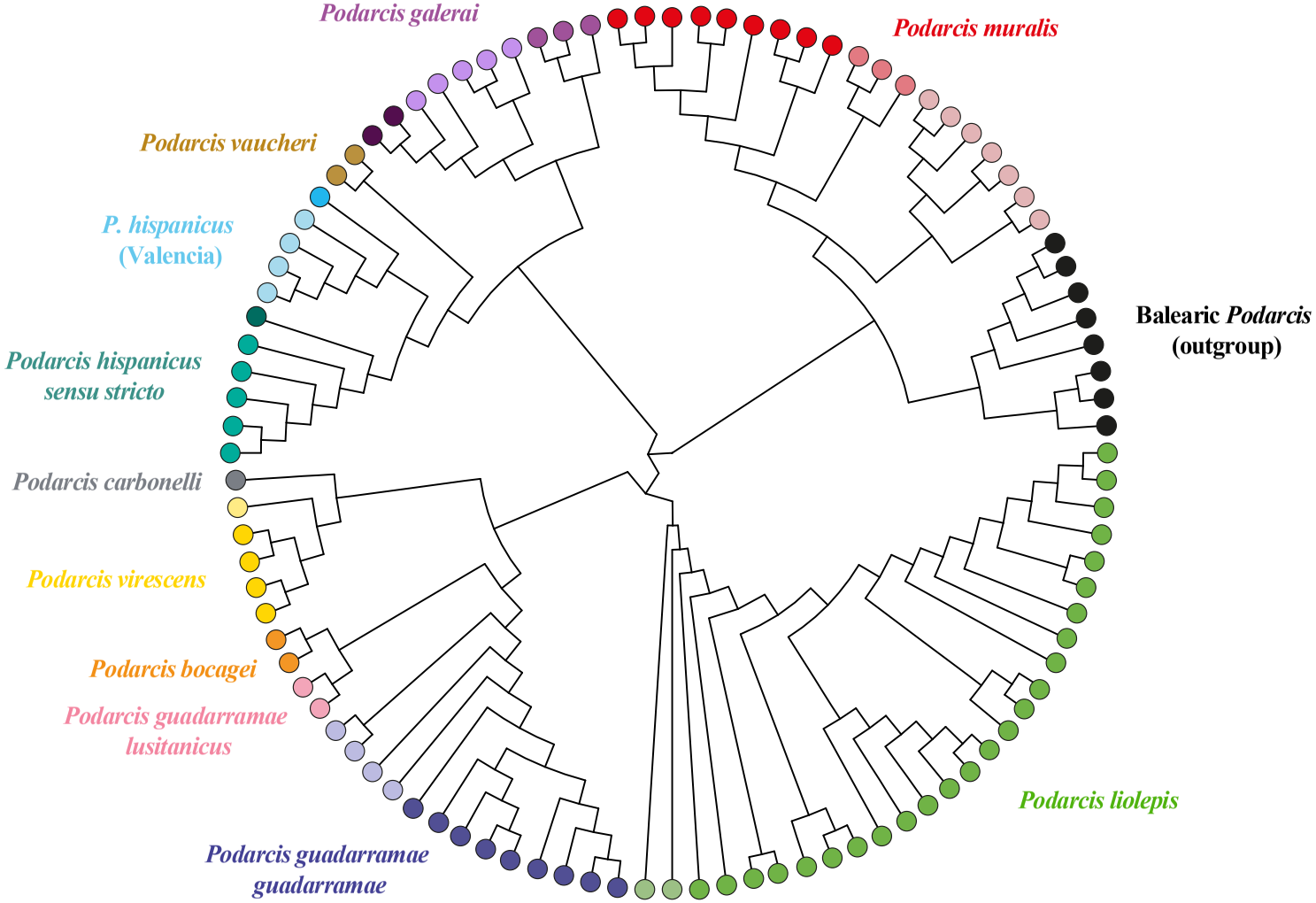
Additional Table 3. Genetic diversity parameters and neutrality tests (Tajima's D and Fu and Li's F statistics) for MC1R (663 bp) phased nuclear gene from all the *Podarcis* popualtions from Iberian Peninsula and North African used in this study. n.s.: non-significant, (*): P<0.05, (**): P<0.02

Species	Number of sequences (N)	Number of segregating sites (S)	Number haplotypes (h)	Haplotype diversity (Hd)	Number of differences pairwise (K)	Nucleotide diversity (Pi)	Tajima's D (1989)	Fu and Li's F (1993)
P. vaucheri	4	7	4	1.000±0.177	3.833	0.0058±0.0016	0.0389$^{n.s.}$	0.0379$^{n.s.}$
P. bocagei	6	0	1	-	-	-	-	-
P.g.guadarramae	32	7	6	0.534±0.088	1.204	0.0018±0.0004	-0.8949$^{n.s.}$	-1.0107$^{n.s.}$
P. g. lusitanicus	4	1	2	0.500±0.265	0.500	0.0008±0.0004	-0.6124$^{n.s.}$	-0.4787$^{n.s.}$
P.virescens	18	6	6	0.680±0.109	1.333	0.0020±0.0005	-0.7759$^{n.s.}$	-0.9018$^{n.s.}$
P. galerai	34	5	8	0.838±0.038	1.624	0.0025±0.0002	0.8671$^{n.s.}$	0.4933$^{n.s.}$
P. hispanicus (Valencia)	12	14	9	0.955±0.047	4.212	0.0064±0.0010	-0.3901$^{n.s.}$	-0.1009$^{n.s.}$
P. hispanicus sensu stricto	24	5	6	0.783±0.063	1.391	0.0021±0.0003	0.1133$^{n.s.}$	1.0009$^{n.s.}$
P. liolepis (all)	106	33	51	0.956±0.011	4.888	0.0074±0.0003	-0.7566$^{n.s.}$	-1.3469$^{n.s.}$
P. liolepis (mainland)	38	30	31	0.984±0.011	4.996	0.0075±0.0006	-1.1235$^{n.s.}$	-1.2672$^{n.s.}$
P. liolepis (Columbretes)	68	10	22	0.899±0.024	3.604	0.0054±0.0002	1.9693$^{n.s.}$	1.8753*
P.muralis	56	5	7	0.606±0.056	1.468	0.0022±0.0002	0.8259$^{n.s.}$	-0.3978$^{n.s.}$

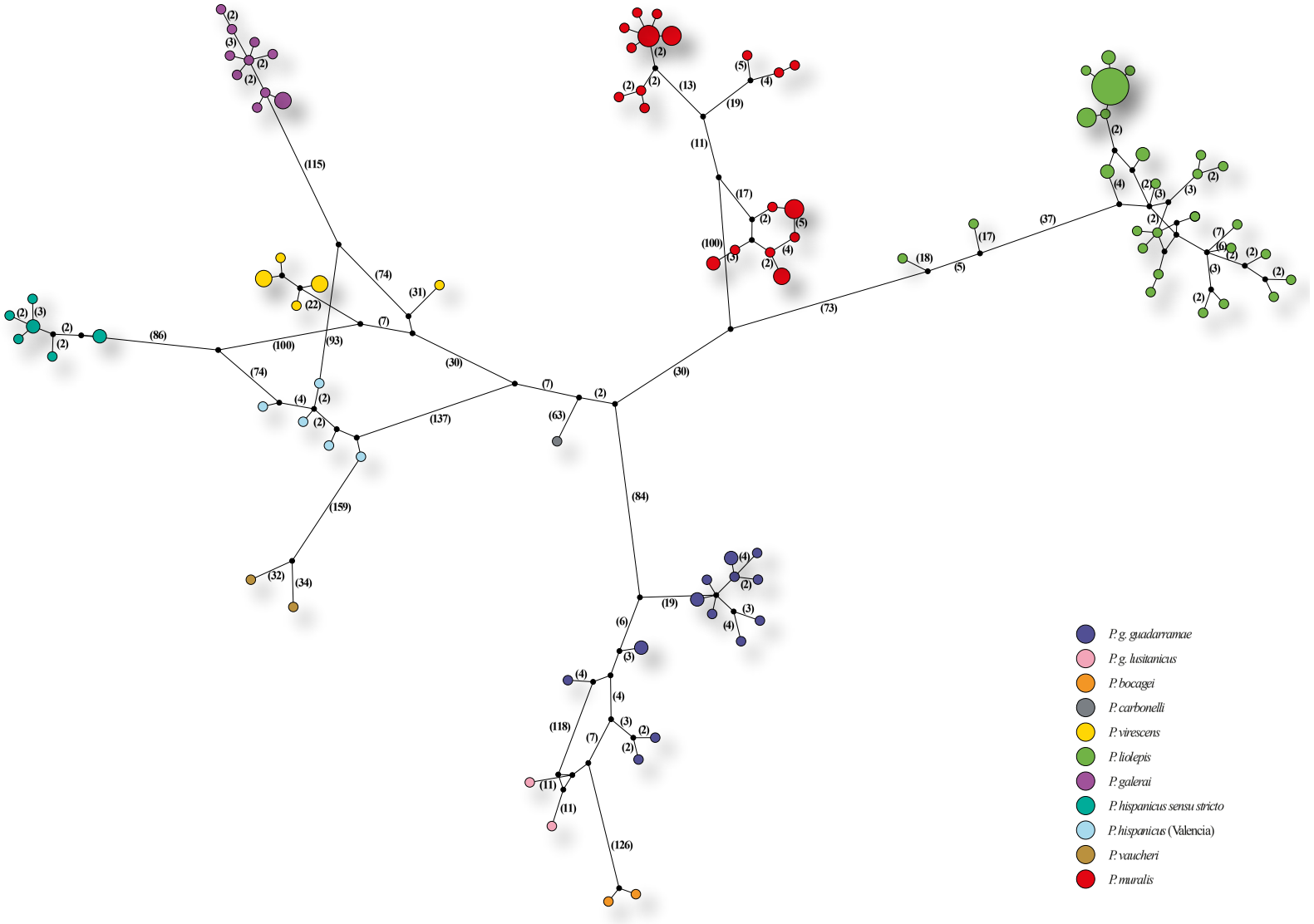
Additional Table 4. Genetic distances (p-distances) of mtDNA concatenated alignment (2,301 bp) of all the *Podarcis* populations from the Iberian Peninsula and North African used in this study.

P. vaucheri										
P. carbonelli	0.0959									
P. bocagei	0.0974	0.0702								
P .g. guadarramae	0.0950	0.0727	0.0663							
P. g. lusitanicus	0.0965	0.0725	0.0617	0.0618						
P. virescens	0.0932	0.0574	0.0809	0.0751	0.0777					
P. h. (Valencia)	0.0855	0.0917	0.0932	0.0978	0.0973	0.0881				
P. h. sensu stricto	0.0902	0.0938	0.1010	0.0988	0.0994	0.0943	0.0730			
P. galerai	0.1030	0.1000	0.0970	0.1022	0.1033	0.0978	0.0925	0.0968		
P. liolepis	0.0977	0.0862	0.0939	0.0971	0.0977	0.0953	0.0981	0.1018	0.1002	
P. muralis	0.1063	0.0978	0.0980	0.0966	0.0966	0.0965	0.0981	0.1023	0.0995	0.0974

Additional Figure 1. Species delimitation of *Podarcis* from the Iberian Peninsula and North African using HierBaps (Tonkin-Hill et al., 2018). Different colours indicated the different clusters obtained. Balearic *Podarcis* was used as outgrou*P.***



Additional Figure 2. TCS haplotype network of *Podarcis* from the Iberian Peninsula and North African based on mtDNA alignment. Mutational steps higher than 1 are indicated with numbers between parentheses, haplotypes circle area is proportional to the number of individuals and the colour identifies the species.



Evolutionary history of wall lizards of the Columbretes archipelago

Introduction

Low levels of genetic diversity in endemic species are generally attributable to the small size of their populations, which is more evident in insular populations where they undergo the effects of fragmentation history on genetic variation in island taxa. Findings demonstrate that geographical distribution of mitochondrial haplotypes evidences historical fragmentation patterns rather than geographic isolation. According to the neutral bottleneck theory, younger islands with larger areas accumulate more nuclear genetic variation than older, smaller islands. Population-specific estimators of genetic differentiation show an inverse correlation with island area, suggesting that smaller islands show greater divergence as a result of their significant susceptibility to drift. Thus, both island area and island isolation period are important predictors of genetic diversity and these patterns probably emerge over progressive fragmentation of ancestral diversity and the consequent cumulative effects of drift, respectively (Hurston et al., 2009).

Although most studies in molecular phylogenetics have used mitochondrial DNA (mtDNA), the causes of discordance between mitochondrial and population histories, including incomplete lineage sorting and introgressive hybridization, are well known. A classical procedure to detect misleading mtDNA relationships is to check for discordance with independent sets of markers (Renoult et al., 2009). Agreement between results obtained with multiple types of markers provides strong support for inferred evolutionary relationships between populations or taxa (Cummings et al., 1995). Conflicting results are also helpful, as they improve our understanding of the historical events that have led to the extant distribution of evolutionary lineages (Shaw, 2002; Morando et al., 2004).

The Columbretes archipelago consists of a group of small islets located in the Mediterranean Sea, covering an area of approximately 19 ha. These uninhabited islets are relatively close to the east Iberian Peninsula (50 km), and to the island of Ibiza (Balearic Archipelago) (100 km). Its volcanic origin is dated between 1-0.3 Ma (Savelli, 2002; Lustrino et al., 2011), which, corresponding to the period after Pleistocene volcanic episodes. Possible connections could have existed between the archipelago and the mainland during the last glaciations, in particular the Würm (mid and low Pleistocene), when the Mediterranean Sea level dropped approximately 120 m. The channel between the Iberian Peninsula and the Columbretes exhibits a depth of approximately 90-100 m (Aparicio et al., 1991; Juan et al., 2004).

The archipelago comprises four different groups of islets (Figure 1): 1) Grossa (13 ha), Mascarat, Senyoreta and Mancolibre, 2) Ferrera (1.5 ha), Espinosa, Bauzá, Valdés and Navarrete; 3) Foradada (1.6 ha), Lobo (0.5 ha) and Méndez Nuñez; 4) Carallot (0.1 ha), Cerquero, Churruca and Baleato. In general, it has remained uninhabited (except for lighthouse keepers who were present from 1855 until 1975). Some islands (Foradada, Ferrera and Carallot) were used for military exercises with live ammunition and it is important to highlight their notorious effects on the flora and fauna of the islands (Castilla & Bauwens, 1991a).

The extant terrestrial vertebrate fauna consists of some breeding bird species and the endemic lizard first named as *Podarcis hispanica atrata*, later elevated as a new species with the name *Podarcis atrata* (Castilla et al., 1998a, 1998b) and recently considered conspecific with *Podarcis liolepis* (Harris et al., 2002, Harris & Sá-Sousa, 2002, Pinho, 2006, 2007, 2008, Renoult et al., 2010, Kaliontzopoulou et al., 2011). The *Podarcis* species is present on only four of the islets that making up the archipelago: Grossa, Mancolibre, Foradada and Lobo. All of them were sampled in this study.

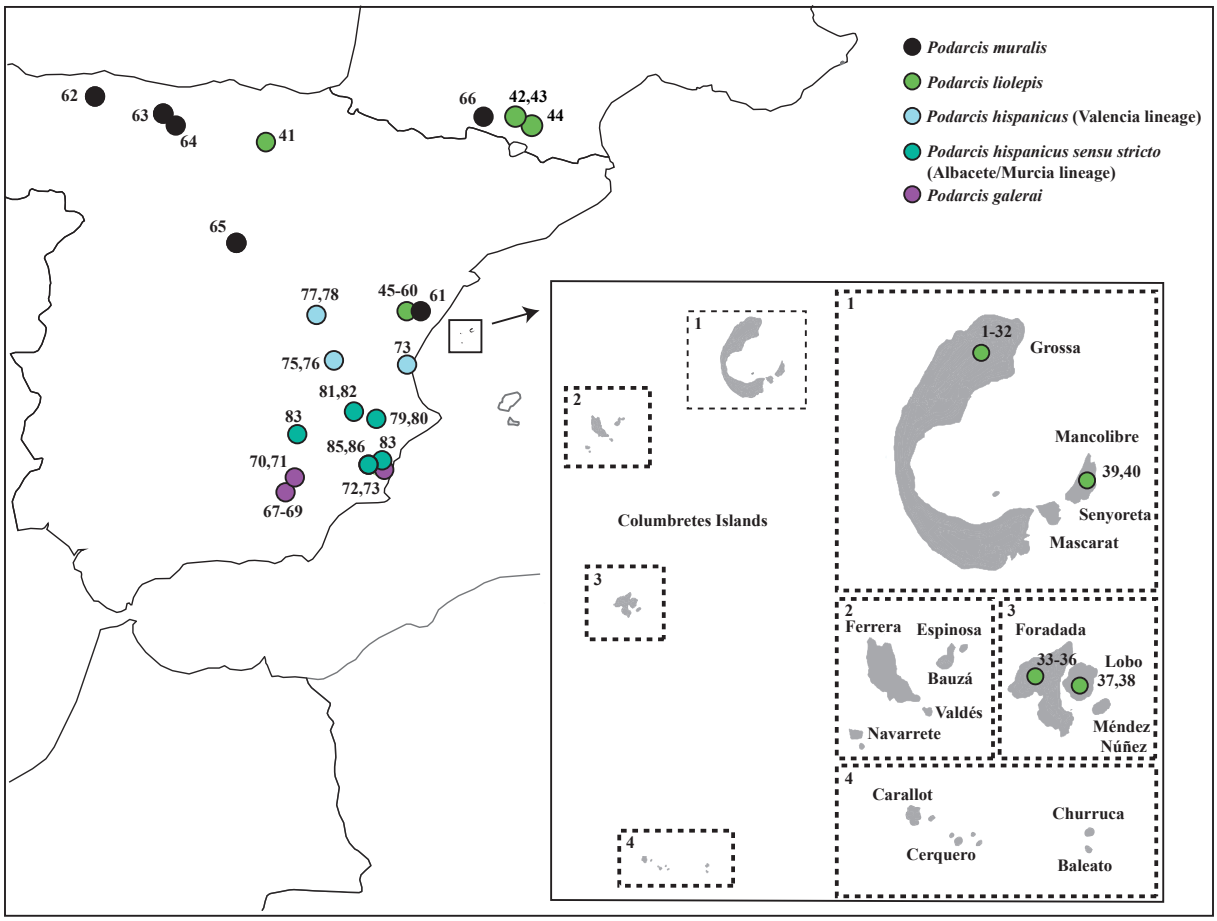


Figure 1. Location of all samples used in this study. Numbers correspond to those in Table 1 and colours to different species indicated in the legend.

It has been postulated that their ancestor may have arrived on the Columbretes islands from the mainland 20,000 years ago, during the last glaciation (Würm) (Castilla et al., 1998a). The unique genetic study on the genetic diversity of the Columbretes archipelago dates back to 1998 (Castilla et al., 1998a) and was based only on a 306 bp fragment of mitochondrial cytochrome b, amplified by means of universal primers (Kocher et al., 1989). Although it refers to the insular *Podarcis* as a new species (*P. atrata*), genetic differentiation suggests that populations from Grossa and Mancolibre are less differentiated than those of Foradada and Lobo and seem to have retained mainland haplotypes whose origin is not clearly established.

The fauna of the archipelago suffered a dramatic episode during the construction of the lighthouse of Grossa Island in 1855, with a drastic campaign of extermination of the snakes (*Vipera latastei*) that included complete brush fire. Fortunately, in 1988 and 1990, the Columbretes Islands were declared a natural and marine reserve, respectively. Although the conservation status of this insular species of *Podarcis* is still not well defined following its recent taxonomic change, it is considered vulnerable by

national institutions (Castilla & Bauwens, 1991a, 1991b). Although the effects that the fire destined to eliminate the vipers caused in the populations of lizards have not been studied yet, attention has been drawn to the high fragmentation of the different populations, reduced habitat, and low number of individuals, characteristics that make this species susceptible to extinction (Castilla et al., 2002, 2006).

Our purpose was to understand the degree of diversity intra archipelago, the possible origin of the founders of the Columbretes lizards, and genetic differentiation between lizard species from Columbretes and their sister taxa from the mainland. It would be especially interesting to be able to assess the effects of the provoked fire in the 19th century on lizard population diversity.

Analyses based on mtDNA and nuclear markers, including individuals from different populations, would contribute to improve the knowledge of the Columbretes *Podarcis* and therefore reveal new insights into their biogeographical history, enabling more concrete taxonomic proposals to be made and consequently providing important information for conservation bodies.

Materials and methods

Sampling

Lizards were caught by careful noosing in their natural habitats. A total of 32 individuals from Grossa Island, four from Foradada, two from Lobo, and two from Mancolibre were sampled in the summers of 2016 and 2017. Tail tips were removed and stored immediately in 100% ethanol. All lizards were released at the same point of capture. Samples and their locations are described in Table 1 and Figure 1.

DNA Amplification and sequencing

A standard phenol-chloroform protocol was used for DNA extraction (Sambrook et al., 1989). A mtDNA fragment providing an alignment of 2,301 bp length was obtained for 20 specimens from Columbretes islands, including partial 12S rRNA, partial cytochrome b (CYTB), partial control region (CR), and two partial subunits of the NADH dehydrogenase gene and associated tRNAs (referred to as ND1, ND2, $tRNA_{Ile}$, $tRNA_{Gln}$, and $tRNA_{Met}$). Primers and amplification conditions are the same as those used in our previous studies of *Podarcis* (Rodríguez et al., 2013, 2014, 2017a; Terrasa et al., 2009a). In addition, 52 individual mtDNA sequences were used from GenBank (Bassitta et al., 2020) (Table 1): eight from Columbretes; 18 *P. liolepis* (from Peñagolosa, South of France, La Rioja); 20 belonging to the *P. hispanicus* complex from Valencia lineage, *P. h. sensu stricto* (Albacete/Murcia lineage), and *P. galerai* (Galera lineage); and six *P. muralis* (from Asturias, France, Castilla, Cantabria, la Pinilla, and Castellón).

Additionally, a cytochrome oxidase I (COI) fragment (657 bp) was amplified by means of primers LCO1490: GGTCAACAAATCATAAAGATATTGG and HCO2198: TAAACTTCAGGGTGACCAAAAAATCA (Folmer et al., 1994). Amplification reaction was made through 35 cycles after initial denaturalization of 4 minutes: 30 seconds at 94ºC, 30 seconds a 48ºC, and 1 minute at 72ºC, followed by a final extension at 72ºC for ten minutes. A total of 26 individuals were studied in this case (Table 1): ten of them from Columbretes (including one from Lobo, Mancolibre, and Foradada); ten *P. liolepis* from the Peñagolosa area; one from Rioja; three from the south of France. Two COI sequences were used from GenBank, one *P. liolepis* from Catalonia, Palamos (accession number MN015111.1) and one *P. muralis* from Panticosa, Spain (accession number MN015063.1) (Garcia-Porta et al., 2019).

Furthermore, four partial nuclear genes were amplified and sequenced: i) melanocortin 1 receptor (MC1R) (663 bp) (8 samples), ii) recombination activating gene 1 (RAG1) (939 bp) (6 samples), iii) apolipoprotein B gene APOBE28 (489 bp) (25 samples) and iv) transcription factor gene KIAA2018 (623 bp) (24 samples). Primers and conditions are described in our previous work (Buades et al., 2013; Rodríguez et al., 2017a). Additional sequences of MC1R and RAG1, previously genotyped in Bassitta et al. (2020), were also included (Table 1).

Both strands of all PCR products were sequenced using a BigDye® Terminator v. 3.1 Cycle sequencing kit (Applied Biosystems, CA, USA) and then genotyped in an automated ABI 3130 sequencer (Applied Biosystems). Sequences were edited using BioEdit v.7.0.5.2. (Hall, 1999) and aligned in the MAFFT v7.423 online server (Katoh et al., 2017) using the iterative refinement method (FFT-NS-i). For protein-coding genes, alignments were verified by translating nucleotide sequences to amino acids. Nuclear data were phased using the PHASE algorithm (Stephens et al., 2001) within DnaSP v.6 (Librado & Rozas, 2009).

Table 1. Species assignation, code number, geographic location and GenBank Accession number of all samples used in this study. NADH=ND1+ND2+tRNAs ($tRNA_{Ile}$, $tRNA_{Gln}$, and $tRNA_{Met}$).

	Species	Code	Country	Locality	Lat	Long	12S	CR	CYTB	NADH	COI	RAG1	MC1R	KIAA2018	APOBE28
1	*P. liolepis*	AB0	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651630	MN651682	MW135433	MN651888	MN651765		
2	*P. liolepis*	AB1	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651631	MN651683		MN651889	MN651766	+	+
3	*P. liolepis*	AB2	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683		MN651890	MN651767		
4	*P. liolepis*	AB3	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683	MW135434	MN651891	MN651768	+	+
5	*P. liolepis*	AB4	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651684	MW135433	MN651892	MN651769	+	+
6	*P. liolepis*	AB5	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651685		MN651893	MN651770	+	+
7	*P. liolepis*	AB6	Spain	Grossa, Columbretes Islands	39.898	0.686							+		
8	*P. liolepis*	AB7	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651633	MN651684	MW135433	MN651894	MN651771	+	+
9	*P. liolepis*	AB10	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683		MN651895	MN651772		
10	*P. liolepis*	AB11	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651631	MN651683		MN651896	MN651773		
11	*P. liolepis*	AB12	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651634			MN651897	MN651774		
12	*P. liolepis*	AB13	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683		MN651898	MN651775		+
13	*P. liolepis*	AB14	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683			+		
14	*P. liolepis*	AB22	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651630	MN651682		MN651899	MN651776		
15	*P. liolepis*	AB32	Spain	Grossa, Columbretes Islands	39.898	0.686							+		
16	*P. liolepis*	AB33	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683		MN651900	MN651777	+	+
17	*P. liolepis*	AB44	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651631	MN651683		MN651901	MN651778	+	+
18	*P. liolepis*	AB52	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683		MN651902	MN651779		
19	*P. liolepis*	AB55	Spain	Grossa, Columbretes Islands	39.898	0.686		MN651585	MN651632	MN651683		MN651903	MN651780		
20	*P. liolepis*	AB62	Spain	Grossa, Columbretes Islands	39.898	0.686							+		
21	*P. liolepis*	AB66	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683			+		
22	*P. liolepis*	AB72	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683			+		
23	*P. liolepis*	AB77	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1		MN651632			MN651904	MN651781		
24	*P. liolepis*	AB88	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651684		MN651905	MN651782		
25	*P. liolepis*	AB001	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683	MW135433	MN651906	MN651783		
26	*P. liolepis*	AB002	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683			+		
27	*P. liolepis*	AB007	Spain	Grossa, Columbretes Islands	39.898	0.686						+			
28	*P. liolepis*	AB008	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651631	MN651683			+		
29	*P. liolepis*	AB009	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651633	MN651683		MN651907	MN651784		
30	*P. liolepis*	AB012	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683		+			
31	*P. liolepis*	AB017	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651635	MN651683	MW135433	+			
32	*P. liolepis*	AB022	Spain	Grossa, Columbretes Islands	39.898	0.686	KT030702.1	MN651585	MN651632	MN651683		+			
33	*P. liolepis*	AF1	Spain	Foradada, Columbretes Islands	39.875	0.671	KT030702.1	MN651585	MN651636			MN651908	MN651785		
34	*P. liolepis*	AF2	Spain	Foradada, Columbretes Islands	39.875	0.671	KT030702.1	MN651585	MN651636	MN651682	MW135433	MN651909	MN651786	+	
35	*P. liolepis*	AF3	Spain	Foradada, Columbretes Islands	39.875	0.671					MW135433	+			
36	*P. liolepis*	AF4	Spain	Foradada, Columbretes Islands	39.875	0.671						+			
37	*P. liolepis*	AO2	Spain	Lobo, Columbretes Islands	39.875	0.672	KT030702.1	MN651585	MN651636			MN651910	MN651787		
38	*P. liolepis*	AO3	Spain	Lobo, Columbretes Islands	39.875	0.672	KT030702.1	MN651585	MN651636	MN651682	MW135433	MN651911	MN651788	+	+
39	*P. liolepis*	AM1	Spain	Mancolibre, Columbretes Islands	39.895	0.690	KT030702.1	MN651585	MN651632			MN651912	MN651789		
40	*P. liolepis*	AM2	Spain	Mancolibre, Columbretes Islands	39.895	0.690	KT030702.1	MN651585	MN651632	MN651686	MW135433	MN651913	MN651790	+	+
41	*P. liolepis*	HL1	Spain	Sazajarra, Rioja	42.588	-2.961	KT030703.1	KT030698.1	MN651637	KT030692.1	MW135441	MN651914	MN651791	+	+
42	*P. liolepis*	PL3	France	Pêch de Foix	42.963	1.623	MN649229	MN651586	MN651638	MN651687	MW135443	MN651915	MN651792	+	+
43	*P. liolepis*	PL36	France	Pêch de Foix	42.963	1.623					MW135443			+	+
44	*P. liolepis*	PL60	France	Vaychis	42.748	1.840	KT030702.1	MN651587	MN651639	MN651682	MW135442	MN651916	MN651793	+	+
45	*P. liolepis*	HF9	Spain	Peñagolosa (Fonte Nova), Castellon	40.245	-0.376	KT030702.1	KT030697.1	KT030685.1	KT030691.1		MN651917	MN651794	+	+
46	*P. liolepis*	HJ2	Spain	Peñagolosa, Castellon	40.245	-0.376	KT030702.1	MN651587	KT030684.1	KT030690.1	MW135435	MN651918	MN651795	+	+
47	*P. liolepis*	HJ3	Spain	Peñagolosa, Castellon	40.245	-0.376	KT030702.1	MN651587	MN651640	MN651688	MW135436	MN651919	MN651796	+	+
48	*P. liolepis*	HJ4	Spain	Peñagolosa, Castellon	40.245	-0.376	KT030702.1	MN651587	MN651641	MN651689		MN651920	MN651797	+	+
49	*P. liolepis*	HPC8	Spain	Peñagolosa, Castellon	40.245	-0.376	KT030702.1	MN651587	MN651640	MN651690	MW135439	MN651921	MN651798		+
50	*P. liolepis*	MPC5	Spain	Peñagolosa (cumbre), Castellon	40.245	-0.376	KT030702.1	MN651587	KT030707.1	KT030709.1	MW135440	MN651922	MN651799	+	+

51	*P. liolepis*	MPC6	Spain	Peñagolosa (cumbre), Castellon	40.245	-0.376	KT030702.1	MN651588	MN651642	MN651690	MW135436	MN651923	MN651800	+	+
52	*P. liolepis*	HP118	Spain	Peñagolosa (Centro de interpretación), Castellon	40.245	-0.376	KT030702.1	MN651587	MN651643	MN651691		MN651924	MN651801		
53	*P. liolepis*	HP218	Spain	Peñagolosa (Centro de interpretación), Castellon	40.245	-0.376	KT030702.1	MN651587	MN651640	MN651692	MW135436	MN651925	MN651802		
54	*P. liolepis*	HP3	Spain	Peñagolosa (Centro de interpretación), Castellon	40.245	-0.376	MN649230	MN651587	MN651644	MN651693	MW135437	MN651926	MN651803		
55	*P. liolepis*	HP4	Spain	Peñagolosa (Centro de interpretación), Castellon	40.245	-0.376	KT030702.1	MN651587	MN651645	MN651694		MN651927	MN651804		
56	*P. liolepis*	HP5	Spain	Peñagolosa (localidad 2), Castellon	40.245	-0.376	KT030702.1	MN651589	MN651646	KT030690.1		MN651928	MN651805		
57	*P. liolepis*	HP8	Spain	Peñagolosa (localidad 3), Castellon	40.245	-0.376	KT030702.1	MN651585	MN651642	MN651690	MW135436	MN651929	MN651806		
58	*P. liolepis*	HP9	Spain	Peñagolosa (cumbre), Castellon	40.245	-0.376	KT030702.1	MN651587	MN651647	MN651690		MN651930	MN651807		
59	*P. liolepis*	HP10	Spain	Peñagolosa (cumbre), Castellon	40.245	-0.376	KT030702.1	MN651587	MN651641	KT030709.1	MW135438	MN651931	MN651808		
60	*P. liolepis*	HP11	Spain	Peñagolosa (cumbre), Castellon	40.245	-0.376	KT030702.1	MN651590	MN651648	KT030690.1	MW135436	MN651932	MN651809		
61	*P. muralis*	Mpfn7	Spain	Peñagolosa (Fonte Nova), Castellon	40.245	-0.376	MN649231	MN651591	MN651649	MN651695		MN651933	MN651810		
62	*P. muralis*	MLL1	Spain	Villaviciosa, Asturias	43.482	-5.433	MN649232	MN651592	MN651650	MN651697		MN651935	MN651812	+	+
63	*P. muralis*	MPG2	Spain	Pto San Glorio, Cantabria	43.067	-4.766	MN649232	MN651593	MN651651	MN651699		MN651939	MN651816	+	+
64	*P. muralis*	MSP1	Spain	Cervera, Castilla y León	42.867	-4.500	MN649232	MN651593	MN651651	MN651701		MN651944	MN651821	+	+
65	*P. muralis*	MLP1	Spain	La Pinilla, Castilla y León	41.222	-3.479	KT030712.1	KT030710.1	KT030706.1	KT030708.1		MN651949	MN651826	+	+
66	*P. muralis*	MP2	France	Moulis	42.960	1.092	MN649233	MN651594	MN651654						
67	*P. galerai*	Gale1	Spain	Galera, Granada	37.739	-2.563	MN649225	MN651579	MN651613	MN651672		MN651869	MN651740		
68	*P. galerai*	Gale3	Spain	Galera, Granada	37.739	-2.563	MN649225	MN651579	MN651613	MN651672		MN651870	MN651741		
69	*P. galerai*	Gale5	Spain	Galera, Granada	37.739	-2.563	MN649225	MN651579	MN651613	MN651672		MN651871	MN651742		
70	*P. galerai*	Df1	Spain	Puebla de Don Fadrique, Granada	37.911	-2.398	MN649225	MN651580	MN651613	MN651672		MN651872	MN651743		
71	*P. galerai*	Df3	Spain	Puebla de Don Fadrique, Granada	37.911	-2.398	MN649225	MN651581	MN651613	MN651672			MN651744		
72	*P. galerai*	Ph16	Spain	Orihuela, Alicante	38.085	-0.948	MN649225	MN651580	MN651614	MN651673		MN651873	MN651745		
73	*P. galerai*	Ph22	Spain	Embalse de la Pedrera, Orihuela	38.032	-0.870	MN649226	MN651580	MN651614	MN651673			MN651747		
74	Valencia lineage*	HB2	Spain	Burjassot, Valencia	39.511	-0.414	KT030701.1	KT030695.1	MN651619	KT030689.1		MN651877	MN651752		
75	Valencia lineage*	PHP3	Spain	Puebla Salvador, Cuenca	39.563	-1.675	MN649227	MN651582	MN651620	MN651677		MN651878			
76	Valencia lineage*	PHP4	Spain	Puebla Salvador, Cuenca	39.563	-1.675	MN649227	MN651582	MN651621	MN651678		MN651879	MN651753		
77	Valencia lineage*	PHCE2	Spain	Ciudad Encantada, Cuenca	40.208	-2.011	KT030701.1	MN651582	MN651622	KT030689.1		MN651880	MN651754		
78	Valencia lineage*	PHCE4	Spain	Ciudad Encantada, Cuenca	40.208	-2.011	KT030701.1	MN651582	MN651623	MN651679		MN651881	MN651755		
79	*P. hispanicus sensu stricto*	Alb1	Spain	Sierra de la Oliva, Albacete	38.765	-0.971	MN649228	MN651583	MN651624	MN651680			MN651756		
80	*P. hispanicus sensu stricto*	Alb2	Spain	Sierra de la Oliva, Albacete	38.765	-0.971	MN649228	MN651583	MN651624	MN651680			MN651757		
81	*P. hispanicus sensu stricto*	Alb3	Spain	Montealegre del Castillo, Albacete	38.826	-1.341	MN649228	MN651583	MN651625	MN651680		MN651882	MN651758		
82	*P. hispanicus sensu stricto*	Alb4	Spain	Montealegre del Castillo, Albacete	38.826	-1.341	MN649228	MN651584	MN651626	MN651680		MN651883	MN651759		
83	*P. hispanicus sensu stricto*	Alb6	Spain	Cañada del Provencio, Albacete	38.518	-2.353	MN649228	MN651583	MN651627	MN651680		MN651884	MN651760		
84	*P. hispanicus sensu stricto*	Seg2	Spain	Sierra Callosa del Segura, Alicante	38.123	-0.895	MN649228	MN651583	MN651628	MN651680		MN651885	MN651761		
85	*P. hispanicus sensu stricto*	Cmp1	Spain	Laderas del Campillo, Murcia	38.060	-1.092	MN649228	MN651583	MN651629	MN651681		MN651886	MN651762		
86	*P. hispanicus sensu stricto*	Cmp4	Spain	Laderas del Campillo, Murcia	38.060	-1.092	MN649228	MN651583	MN651629	MN651681			MN651764		
	Outgroup**														
	P. lilfordi	D22	Spain	Dragonera, Balearic Islands	39.584	2.320	EF694761.1	EF694773.1	KC623944.1	EU006730.1					
	P. lilfordi	Cr1	Spain	Es Colomer, Balearic Islands	39.945	3.131	EF694760.1	EF694775.1	EF694801.1 / EF990524.1	EU006737.1					
	P. lilfordi	Cp1	Spain	Cabrera harbour, Balearic Islands	39.151	2.934	EF694764.1	EF694780.1	KF003361.1 /EF990531.1	EU006743.1					
	P. lilfordi	AG1	Spain	Addaia Gran, Balearic Islands	40.016	4.210	EF694766.1	EF694786.1	EF694809.1 / EF694826.1	EU006756.1					
	P. pityusensis	Alg1	Spain	Alga, Balearic Islands	38.977	1.532	EF694768.1	EF694794.1	JX852075.1	JX852119.1					
	P. pityusensis	Eiv1	Spain	Ibiza, Balearic Islands	38.907	1.421	EF694768.1	EF694794.1	JX852050.1	JX852121.1					
	P. pityusensis	Fort4	Spain	Formentera, Balearic Islands	38.753	1.436	EF694768.1	EF694794.1	JX852075.1	JX852121.1					

***Valencia lineage belongs to *P. hispanicus* complex.**

****Brown et al., 2008; Terrasa et al., 2009a; Buades et al., 2013; Kapli et al., 2013; Rodríguez et al., 2013.**

Divergence and phylogenetic analyses

Basic genetic diversity parameters were calculated with DnaSP v.6 (Librado & Rozas, 2009) for concatenated mitochondrial alignment, COI fragment, and each phased nuclear gene using only Columbretes Island samples (Table 2). DNaSP was also used to calculate Tajima's D (Tajima, 1989) neutrality statistic, which contrasts estimates of θ based on segregating sites (S) and pairwise differences (k) to determine deviation from selective neutrality. Pairwise mismatch distribution to test for population expansion (Rogers & Harpending, 1992) was carried out using DNaSP.

Best-fit nucleotide substitution models and partitioning scheme were chosen simultaneously using PartitionFinder V1.1.1 (Lanfear et al., 2016) under the Akaike Information Criterion (AIC) for the mtDNA dataset (Table 1). Partitioning schemes were defined by hand with the "user" option, with branch lengths of alternative partitions "unlinked" to search for the best-fit scheme, which consisted of three partitions: non-coding fragments [GTR+I], 1st and 2nd codon of coding regions [HKY+I+G], and 3rd codon of coding regions [GTR+I].

Phylogenetic analyses were performed using Maximum Likelihood (ML) and Bayesian Inference (BI) methods based on mtDNA dataset with seven *Podarcis* from the Balearic Islands used as an outgroup.

Maximum likelihood analyses were performed using IQ-TREE version 1.6.10 (Nguyen et al., 2014). Partitions and the best-fit substitution model were applied and 10^6 bootstrap replicates were conducted based on the ultrafast bootstrap approximation (UFBoot) (Minh et al., 2013; Hoang et al., 2017) for statistical support.

Bayesian analyses were performed with MrBayes 3.2.1 (Ronquist et al., 2012). Analyses were run for 10^7 generations with sampling frequency every 10^3 generations. Numbers of runs and chains were left as default, two and four, respectively. Sufficient number of generations was confirmed by examining the stationarity of the log likelihood (lnL) values of the sampled trees and ensuring the value of average standard deviations of the split frequencies was lower than 0.01. Results were analysed in Tracer v1.7 (Rambaut et al., 2018) to assess convergence and effective sample sizes (ESS) for all parameters. A burn-in of 25% was applied and phylogenetic trees were visualized and edited using Figtree v1.4.2 (Rambaut, 2014).

TCS statistical parsimony network approach (Clement et al., 2000) with 95% connection limit implemented in the program PopART 1.7 (http://popart.otago.ac.nz) (Leigh & Bryant, 2015; Clement et al., 2002) was used to infer i) genealogical relationships between *P. liolepis* from Columbretes Islands and from the mainland based on haplotypes of each phased nuclear gene (RAG1, MC1R, KIAA2018, and APOBE28) and mtDNA alignment, using *P. muralis* as outgroup; and ii) intra-island diversity based on RAG1 nuclear gene and mtDNA alignment.

BEAST v.5 (Heled & Drummond, 2010) was used to simultaneously infer phylogenetic relationships and divergence times between Columbretes *Podarcis* and different lineages from the Iberian Peninsula based on mtDNA sequence dataset (72 individuals) and seven *Podarcis* from the Balearic Islands, used as calibrator. The calibration was specified from a normal distribution (5.32, 0.01) based on knowledge of the timing of the end of the Messinian salinity crisis (5.33 Ma) and the very rapid refilling of the Mediterranean basin that would have separated the two Balearic island *Podarcis* (i.e., *P. lilfordi* and *P. pityusensis*) (see Brown et al., 2008). Partitions and evolutionary models were the same as those used for MrBayes. The BEAST MCMC sampler was run twice for $5x10^8$ generations, with one step per 5,000 sampled. A relaxed log normal clock model was specified, and a Yule model was used for the tree prior.

Table 2. Genetic diversity parameters and neutrality test (Tajima, 1989) for each nuclear gene and for mtDNA alignment and COI gene from Columbretes archipelago samples.

	N (N phased)	bp	Polymorphic positons (S)	Number of haplotypes (h)	Haplotype diversity (Hd)	Nucleotide diversity (pi)	Average nucleotide differences (k)	D Tajima (1989)
Nuclear genes								
RAG1	32 (64)	939	15	12	0.750±0.050	0.0021±0.00020	1.990	-1.100$^{n.s.}$
MC1R	34 (68)	663	10	22	0.899±0.024	0.0054±0.00019	3.604	1.969$^{n.s.}$
KIAA2018	10 (20)	623	14	11	0.884±0.054	0.0076±0.00053	4.716	0.714$^{n.s.}$
APOBE28	10 (20)	489	3	3	0.563±0.063	0.0023±0.00026	1.126	0.886$^{n.s.}$
mtDNA								
CYTB, NADH, CR, 12S	28	2301	11	10	0.770±0.076	0.0009±0.00017	2.106	-0.833$^{n.s.}$
COI	10	657	1	2	0.200±0.154	0.0003±0.00023	0.200	-1.111$^{n.s.}$

n.s.: no significant

Results and discussion

Genetic diversity of Columbretes islands based on mitochondrial and nuclear genes

Only 11 polymorphic sites, of the 28 genotyped specimens from Columbretes archipelago, were detected in the mtDNA fragment sequenced (2,301 bp), corresponding to an extremely low diversity (pi=0.0009). Ten haplotypes were detected, eight in Columbretes Grossa, one present in Mancolibre, and one haplotype shared between Foradada and Lobo. In relation to the COI fragment, the ten individuals sequenced (including seven from Columbretes Grossa and one from Mancolibre, Foradada, and Lobo) shared the same haplotype with the exception of one position in an individual from Columbretes Grossa. This low variability is reflected in the low values of haplotype diversity (Hd=0.200) and nucleotide diversity (pi=0.0003).

These results drastically contrast with those obtained by Castilla et al. (1998a) who observed 95 polymorphic sites, 85 of them parsimony informative, in a fragment of 306 bp of CYTB gene. We compared our CYTB results with the 14 haplotypes from the Columbretes islands deposited in GenBank (accession numbers AJ004987, AJ004994-AJ004996, AJ004910-AJ004911, AJ004990-AJ004992, AJ224407-AJ224409, AF052636-AF052637), resulting in a pattern of variation with numerous changes especially located every three nucleotides. This result is in accord with a non-specific amplification of the mtDNA CYTB gene in Castilla et al. (1998a), probably caused by the use of universal primers (Podnar et al., 2007). Thus, we do not confirm the mtDNA variability previously described in *Podarcis* from the Columbretes archipelago, but rather its extreme homogeneity indicating a recent founder effect at the maternal level or the passage through a recent bottleneck of the population (Table 2). These findings are also corroborated in the expansion analysis where pairwise differences distribution corresponds more to a growth-decline model than to a constant size model (Figure 2).

Four partial nuclear genes (RAG1, KIAA2018, APOBE28 and MC1R) were sequenced comprising a total of 2,714 bp. They also show low values of genetic diversity (Table 2), as haplotype diversity (0.563-0.899) and average of nucleotide differences (1.126-4.716). RAG1 gene is the most diverse gene with 15 haplotypes (phased), followed by KIAA2018 with 14 haplotypes, MC1R with ten haplotypes, and APOBE28, the least diverse, with only three haplotypes.

Phylogenetic relationships between the different islands of the Columbretes archipelago are shown in Figure 3, based on mtDNA genome (Figure 3a) and the nuclear gene RAG1 (Figure 3b), by way of example. In both it is interesting to highlight that Foradada and Lobo share the same haplotype, while the nuclear and mtDNA haplotype of Mancolibre is closer to Grossa Island, just as could be expected, given that Foradada and Lobo, on the one hand, and Grossa and Mancolibre on the other hand, belong to different, geographically distant groups of islands. A maximum of three mutational steps separates both groups of islands. Similar patterns were observed in the three other nuclear genes sequenced.

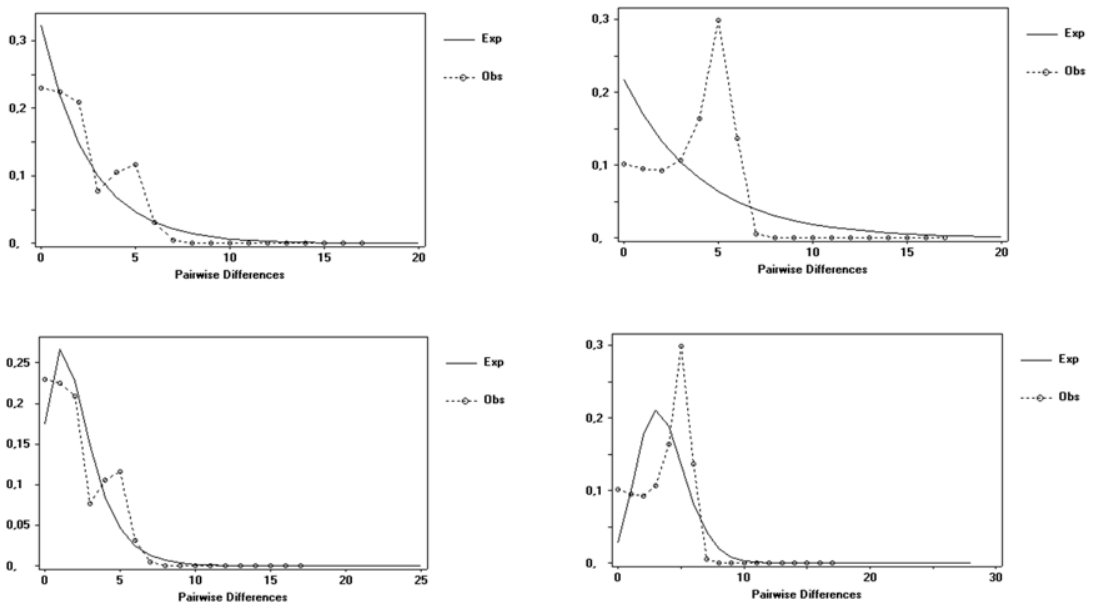


Figure 2. Pairwise mismatch distribution for Columbretes Islands samples based on mtDNA alignment (left) and MC1R nuclear loci (right). Expected frequencies (solid line), based on a constant size (above) and growth-decline model (below) are compared to observed frequencies (dotted line).

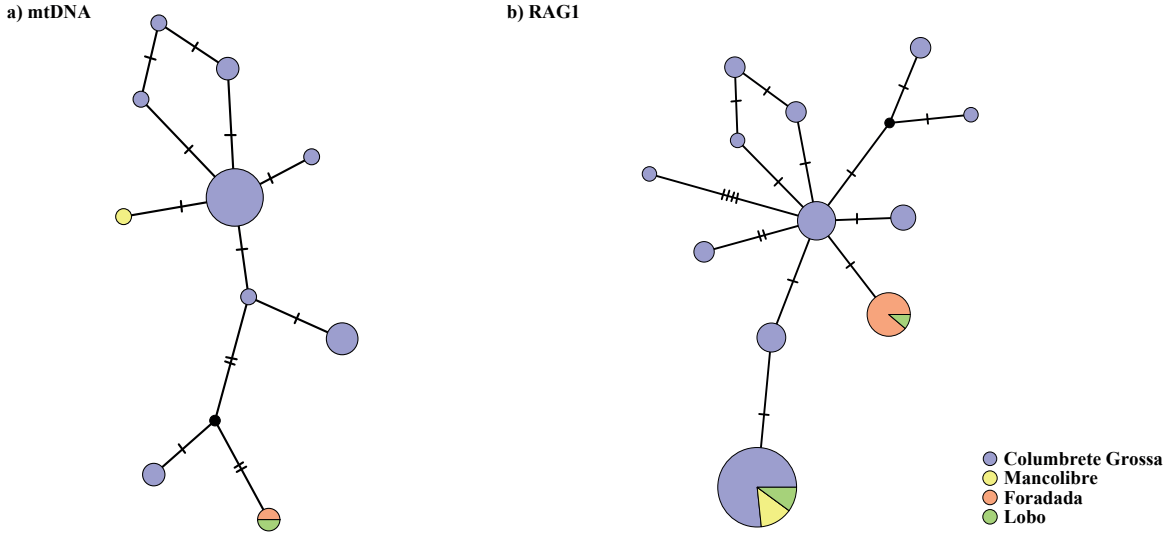


Figure 3. TCS haplotype networks of Columbretes Island samples for mtDNA alignment (a) and for nuclear loci RAG1 (b).

Phylogenetic relationships and possible origin of Columbretes islands wall lizard

The endemic lizard of Columbretes archipelago was first named as *P. hispanica atrata*, was later considered as a new species called *P. atrata* (Castilla et al., 1998b), and has recently been considered conspecific with *P. liolepis*, the typical species of the eastern Iberian Peninsula (Harris & Sá-Sousa, 2002, Renoult et al., 2010, Geniez et al., 2014). Lately, three new lineages have been described in the eastern of Iberian Peninsula area, within that constituted *P. hispanicus* complex: *P.* galerai, *P. hispanicus sensu stricto* (Albacete/Murcia lineage), and *P. hispanicus* (Valencia lineage) (Pinho et al., 2006, Renoult et al., 2009, Kaliontzopoulou et al., 2011, Bassitta et al., 2020).

In our study, samples from Columbretes islands are grouped together and then, in the same cluster, joined to *P. liolepis* from the mainland, specifically from Peñagolosa (Castellon, Spain). The rest of the *P. liolepis* samples from La Rioja and south of France constitute clear separate clades. This pattern is more evident in analyses based on the four mtDNA fragments alignment, for both phylogenetic trees (ML and BI) (Figure 4) and TCS (Figure 5a), than the patterns shown by nuclear genes (Figure 6). The same pattern is revealed in TCS based on the mtDNA COI fragment (Figure 5b) where *P. liolepis* samples from France and from La Rioja and Catalonia in Spain also form a separated cluster with respect to Columbretes and Peñagolosa samples. TCS networks based on nuclear genes (Figure 6) indicate the *P. liolepis* population from Columbretes Islands shares many haplotypes with the conspecific mainland population, confirming the genetic proximity between Columbretes and Peñagolosa lizards.

These results are not in agreement with those obtained by Castilla et al. (1998a, 1998b) who elevated Columbretes lizard population to species rank, because of the high differentiation found between them and samples from the mainland (Valencia). Taking into account the current deeper knowledge of lizards inhabiting the SE region of the Iberian Peninsula (Bassitta et al., 2020), it is likely Castilla et al. (1998a) compared Columbretes samples with populations from one of the other lineages of the *P. hispanicus* complex that inhabit this area, and not *P. liolepis*.

Thus, we can confirm the Columbretes lizards belonging to *P. liolepis* species and the great similarity with the individuals from Peñagolosa, especially at mitochondrial level. These findings also indicate the great diversity present in this *Podarcis* species and that their distribution in the Iberian Peninsula is not yet well defined, so it would be necessary to obtain more samples from more locations so as to obtain a better picture of *P. liolepis* lizard populations throughout its range of distribution.

Time-calibrated phylogeny based on mtDNA (Figure 7) provided a time of divergence between Foradada+Lobo and Grossa+Mancolibre at 1.59 Ma (95% HPD: 2.95-0.52 Ma), coinciding with an interval of time of several sea level fluctuations between glacial and interglacial periods. Time-calibrated tree also showed that the Columbretes Islands population once separated from mainland (Peñagolosa) populations (~1.77 Ma) did not have more contacts and therefore then diverged in isolation. Several events of decrease in diversity (bottleneck) or expansion could have occurred, as corroborated by the growth-decline analysis performed with DnaSP (Figure 2), in the demographic history of Columbretes lizard insular populations. One of these demographic events could have been caused by the effect of the provoked fire that occurred in the 19th century.

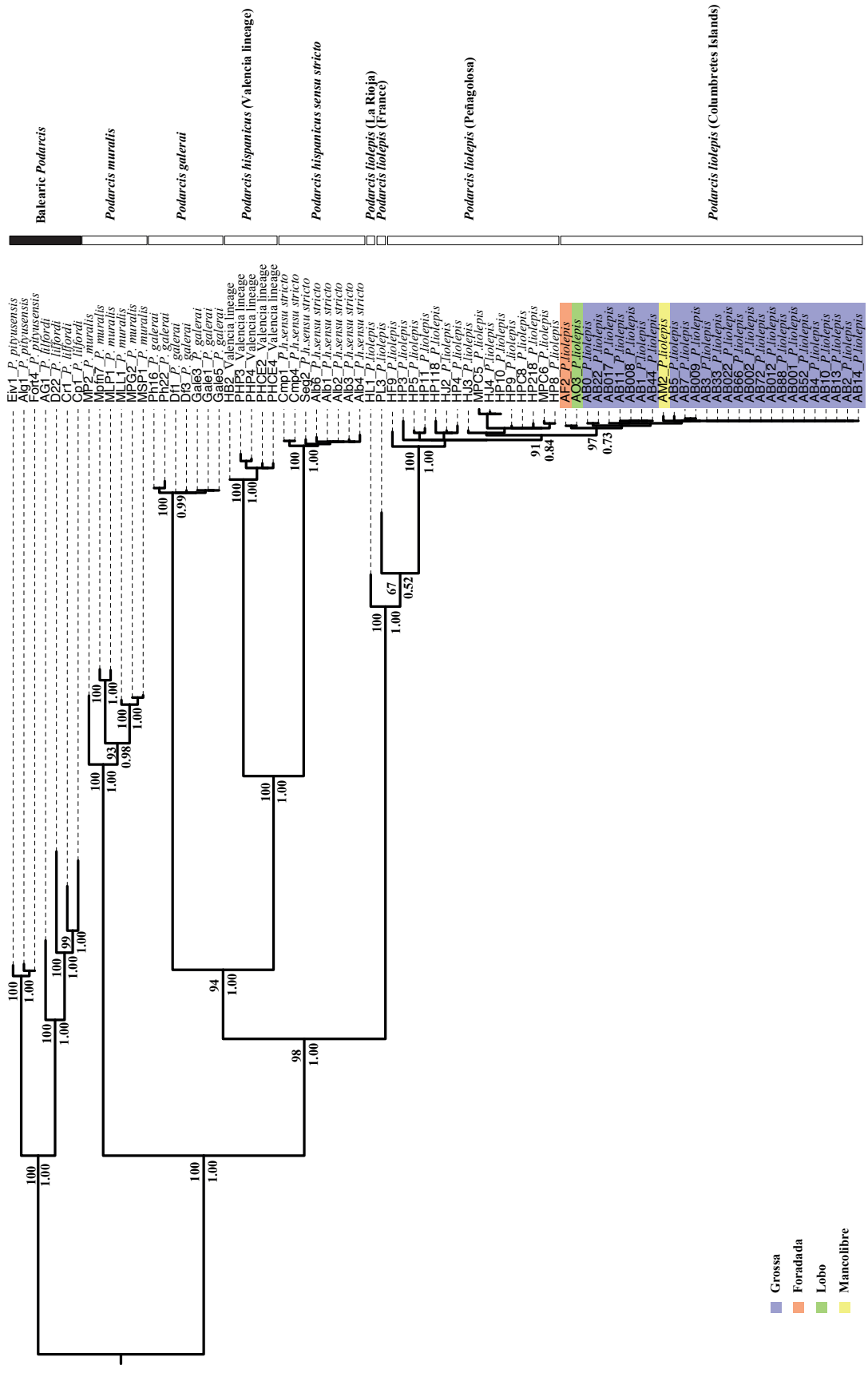


Figure 4. Gene tree based on ML for mitochondrial data showing the close position of Columbretes Island samples to *P. liolepis* from Peñagolosa, Spain. Balearic clade was used as an outgroup. Numbers above branches correspond to bootstrap support and numbers below branches are posterior probabilities from Bayesian analysis.

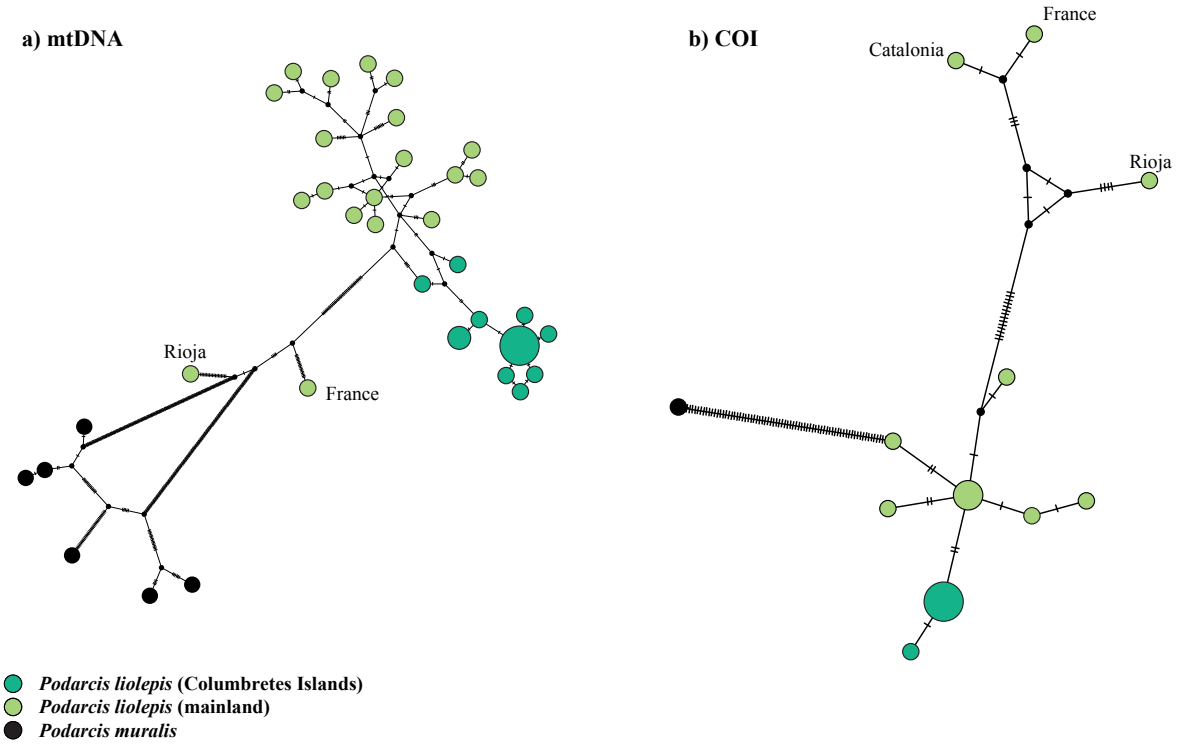


Figure 5. TCS haplotype networks of *P. liolepis* samples from both Columbretes Islands and from the Iberian Peninsula mainland for mtDNA alignment (2,301 bp) (a) and for COI fragment (657 bp) (b). *Podarcis muralis* was included as outgroup.

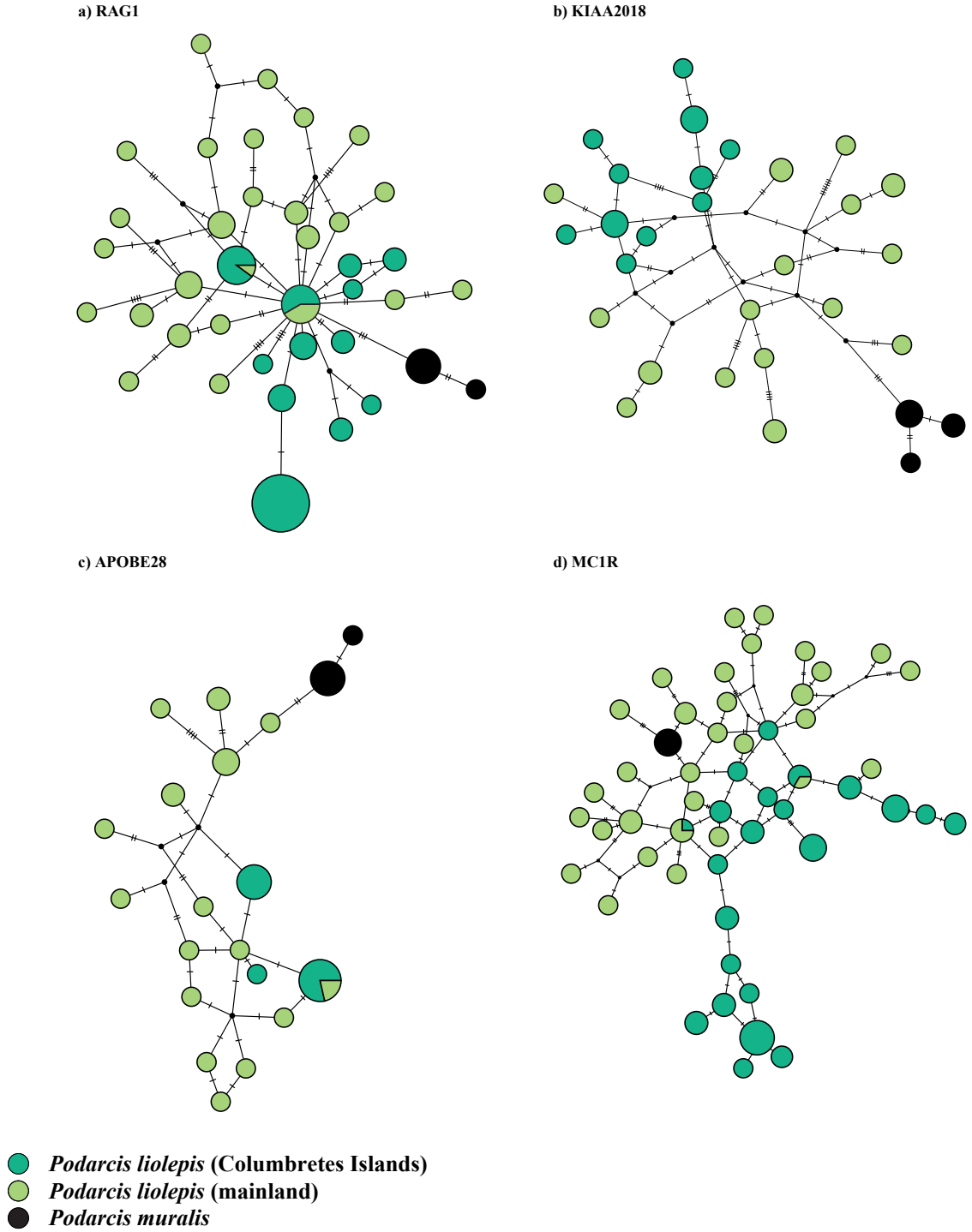


Figure 6. TCS haplotype networks of *P. liolepis* samples from both Columbretes Islands and from the Iberian Peninsula mainland for the four nuclear loci studied: RAG1 (a), KIAA2018 (b), APOBE28, and c) MC1R. *Podarcis muralis* was included as outgroup.

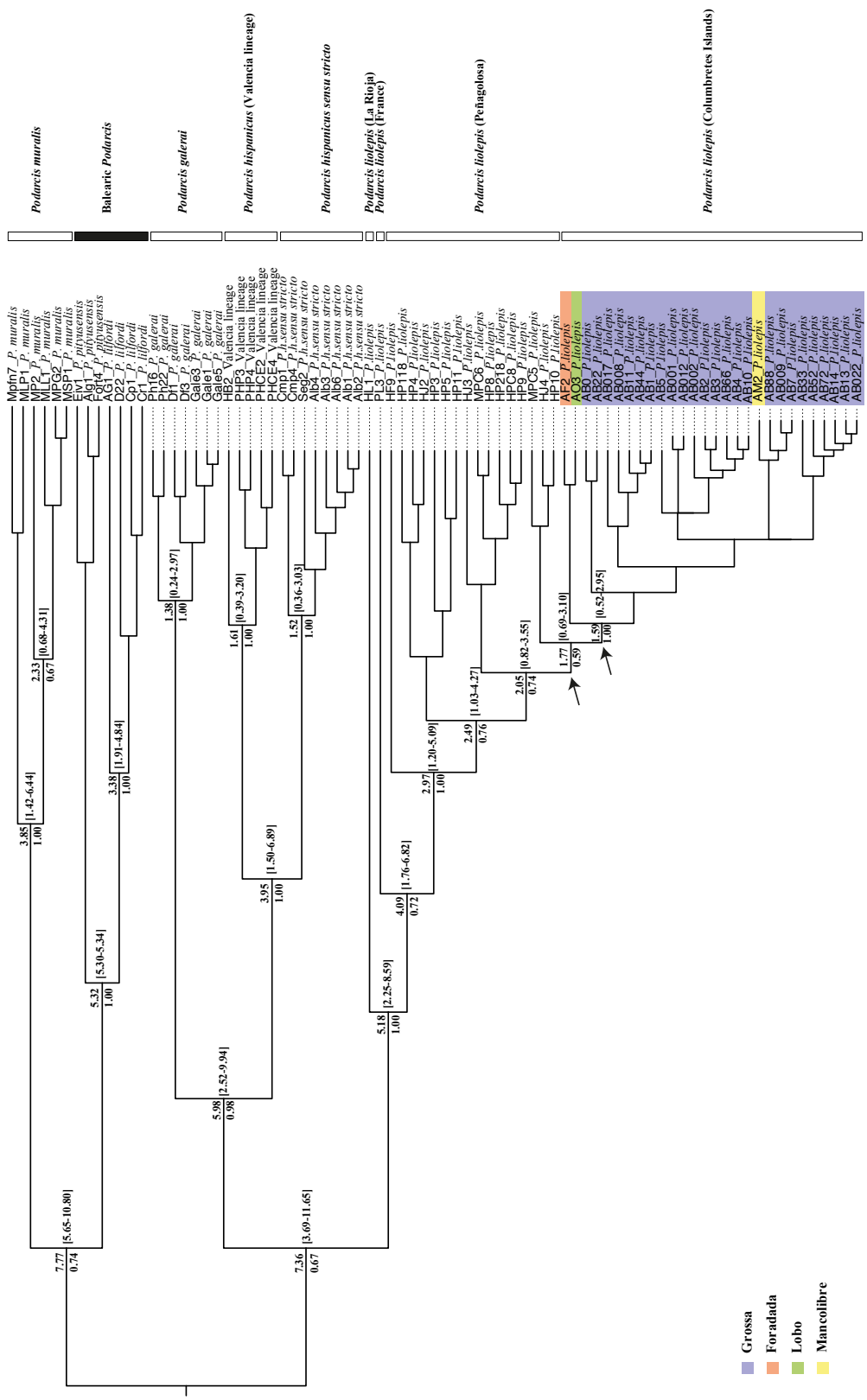


Figure 7. BEAST tree with estimated divergence time based on mtDNA dataset (2,301 bp). Separation of the Balearic *Podarcis* clade was used as a calibration. Numbers at the right of nodes indicate a 95% credible interval of divergence, mean height (above) and posterior probability (below) are shown to the left. Black arrows indicated the time of divergence between *P. liolepis* from Peñagolosa and Columbretes islands samples and the time of divergence between Foradada+Lobo and Grossa+Mancolibre from Columbretes archipelago, respectively.

CHAPTER 2

Endemic lizard from the Balearic Islands: *Podarcis lilfordi*

Genomic signatures of drift and selection driven by predation and human pressure in an insular lizard

Bassitta M; Brown RP; Pérez-Cembranos A; Pérez-Mellado V; Castro JA; Picornell A and Ramon C.

Scientific Reports (in revision)

Morphological and genetic diversity of the Balearic lizard, *Podarcis lilfordi* (Günther, 1874). It is relevant for its conservation?

Pérez-Cembranos A; Pérez-Mellado V; Alemany I; Bassitta M; Terrasa B; Picornell A; Castro JA; Brown RP and Ramon C.

Diversity and Distributions (2020) 26(9):1122-1141

Introduction

In this chapter, the focus is one of the endemic lizards that inhabit the Balearic Islands: *P. lilfordi*. This species is distributed in islets and islands in Menorca, Mallorca, and the Cabrera archipelago. The small population size and geographic isolation of some of these populations result in interesting patterns of diversity and divergence.

The first study, currently in revision (*Scientific Reports*), is a genome-wide approach (ddRADseq) of ten populations of *P. lilfordi* with different characteristics regarding population size and skin pigmentation. Over 72,000 SNPs distributed along the genome were obtained with the aim of discovering the global genetic structure and patterns of diversity and selection of these populations. The results of this work support the combined role of genetic drift and divergent selection in shaping the genetic structure of these endemic island inhabitants. Environmental factors such as human pressure and predation appear to be important drivers of adaptive divergence in these lizard populations.

The second study is a multidisciplinary work that aims to characterise the genetic and morphological diversification of the endemic Balearic lizard *P. lilfordi* and to assess the relevance of this diversity to conservation measures. The genetic approach is based on mitochondrial (genes 12S rRNA, RC, CYTB, NADH, COI) and nuclear specific genes (MC1R). My particular contribution to this study was: a) to assess the genetic diversity on mtDNA by means of a Bayesian analysis and genetic distances (p-distances) using a concatenated mtDNA alignment (2,382 bp); and b) to evaluate the diversification of the nuclear gene (MC1R) on this species by performing a TCS network analysis.

Genomic signatures of drift and selection driven by predation and human pressure in an insular lizard

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Abstract

Double digest restriction-site associated DNA sequencing was performed on 10 selected populations of the endangered Balearic Islands lizard (*Podarcis lilfordi*). The objective was to detect genome-wide single nucleotide polymorphisms (SNP), to know the divergence among populations, to establish the impact of the population size on genetic variability, and to evaluate the role of different environmental factors (biotic capacity, number of vascular plants, predation, human pressure, and presence of rats or seagulls) in local adaptation. Analyses of 72,846 SNPs supported a highly differentiated genetic structure. The populations with the lowest population size (Porros, Foradada and Esclatasang islands) presented a high degree of differentiation, suggesting the effect of genetic drift due to isolation. Outlier tests identified ~2% of loci as high F_{ST} outliers, indicating that divergent natural selection significantly contributes to population divergence. Geographical distribution of the species and environmental factors appear to be fundamental drivers of divergence in lizard populations, with predation and human pressure the most explanatory variables in RDA analysis. These results support the combined role of genetic drift and divergent adaptation, through natural selection, in shaping the genetic structure of these endemic lizard populations.

Key words: *Podarcis lilfordi*; genome-wide; RAD sequencing; genetic drift; natural selection; human pressure, predation pressure.

Introduction

Natural selection is the driving force of Darwinian evolution (Mayr, 1963). However, the detection of unambiguous evidence for natural selection could be elusive, due to the influence of demographic history in populations under study. In islands, the evolutionary mechanism generating this diversity could be associated with the isolation of populations, since it is a characteristic feature related to speciation (Grant, 1998), yet the genetic structure of island populations is also the result of historical events and evolutionary processes such as genetic drift, selection or gene flow (Garant et al., 2007; Armstrong et al., 2018).

Most of the genetic variation found in populations is selectively neutral, originated by random mutations and fixed by genetic drift that may support the maintenance of genetic diversity, particularly in small populations (Wright, 1946; Kimura & Crow, 1964). In the absence of human intervention, gene flow between island is limited or absent, which causes a reduction in genetic variation that can be partially solved through mutation (Eldridge et al., 1999). Nonetheless, morphological divergence and high environmental heterogeneity between islands suggests that divergent selection may also play a key role (Luikart et al., 2003; Nei et al., 2010; Weigelt et al., 2013). Environmental variability can be associated with local adaptation of species (Huey et al., 2000), although some physiological functions may be highly conserved within species (Prates et al., 2013). Thus, it is unclear whether the occurrence of a species in different environments is linked to local adaptation and associated genetic differentiation (Prates et al., 2018). Genetic drift and divergent selection could both drive genetic differentiation and population divergence between islands (Funk et al., 2016; Friis et al., 2018).

As an insular endemism inhabiting a large group of coastal islands of Mallorca and Menorca (Balearic Islands, Spain) (Figure 1), the Balearic lizard, *P. lilfordi*, is a suitable system to study the effect of population size on genetic structure and environmental traits on local adaptation. *Podarcis lilfordi* shows strongly different population sizes (Pérez-Mellado et al., 2008) ranging from fewer than 100 individuals, to over 100,000 individuals, with a large variability of environmental conditions (Pérez-Cembranos et al., 2020). While other systems are characterized by local adaptations linked to climatic differentiation along a large range of distribution (Prates et al., 2018), the range of this species is restricted to a limited geographical area of the Western Mediterranean basin, where climatic characteristics are only slightly variable (Pérez-Cembranos et al., 2020). However, other environmental traits, as food availability, habitat structure, orography, predation pressure, the presence of potential competitors and the human pressure, are extremely different in each population. Thus, a comparison of genetic characteristics of these populations in relation to selected environmental predictors could be of great interest.

Double digest restriction-site associated DNA sequencing (ddRADseq) combines restriction enzymes and next-generation sequencing, thereby enabling the detection of thousands of SNP markers in non-model organisms (Baird et al., 2008; Peterson et al., 2012; Andrews et al., 2016). In this study, ddRADseq was used to reexamine the population history of *P. lilfordi*, previously achieved with mitochondrial DNA (mtDNA) (Terrasa et al., 2009a), and explore how genetic drift and divergent selection, driven by different environmental factors, have shaped genome diversity.

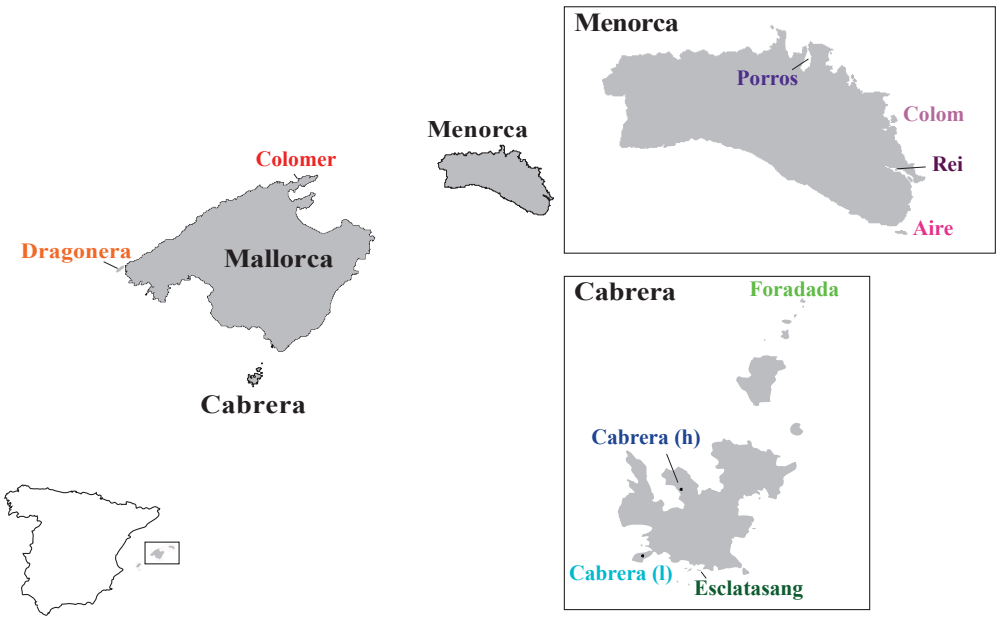


Figure 1. Locations in Mallorca, Menorca and Cabrera islands of all samples used in this study. Cabrera (h): harbour, Cabrera (l): lighthouse

Results

A total of 6.8 billion paired-end reads of 101 bp length were generated from the 91 individuals. After *denovo_map.pl* and filtering steps, 288,286 SNPs called from 80,091 ddRAD contigs were obtained with mean coverage of 28.6 per site. Of these, the first SNP for each locus was obtained (72,846 SNPs), this number is fewer than the number of loci because SNPs that were present in only 20% of individuals were removed.

Population structure

Nucleotide diversity ranged between 0.120 (Porros islet) and 0.182 (Cabrera harbor). Foradada, Esclatasang and Porros presented the highest number of private alleles (746, 475, and 945, respectively) indicating a high genetic differentiation, with no gene flow between them and the other populations, (Brown et al., 2016; Valbuena-Ureña et al., 2017) probably due to their strong geographical isolation. In general, values of inbreeding coefficient (F_{IS}) were low (less than 10%) (Supplementary Table 1), despite the small population size of some populations.

Patterns of divergence based on F_{ST} distance analysis were highly congruent with previous results (Terrasa et al., 2009a; Brown et al., 2008), with Menorca populations showing a clear differentiation respect to Mallorca together with Cabrera populations (Supplementary Figure 1). Taking into account all the SNPs (72,846 SNPs), the highest values were the distance between Porros islet (Menorca) and all the other populations from Mallorca and Cabrera and between the two Cabrera islands (Foradada and Esclatasang) and Menorcan populations. The lowest values were found between the two locations found within Cabrera island (harbour and lighthouse), between Mallorca populations (Dragonera and Colomer) and Cabrera main island, and between Menorcan populations (except for Porros islets). The divergent position of Porros, Foradada and Esclatasang was less pronounced when only outlier SNPs (1,355 SNPs) were considered, while Mallorca populations showed a more divergent position with regard to Cabrera populations (Supplementary Figure 1).

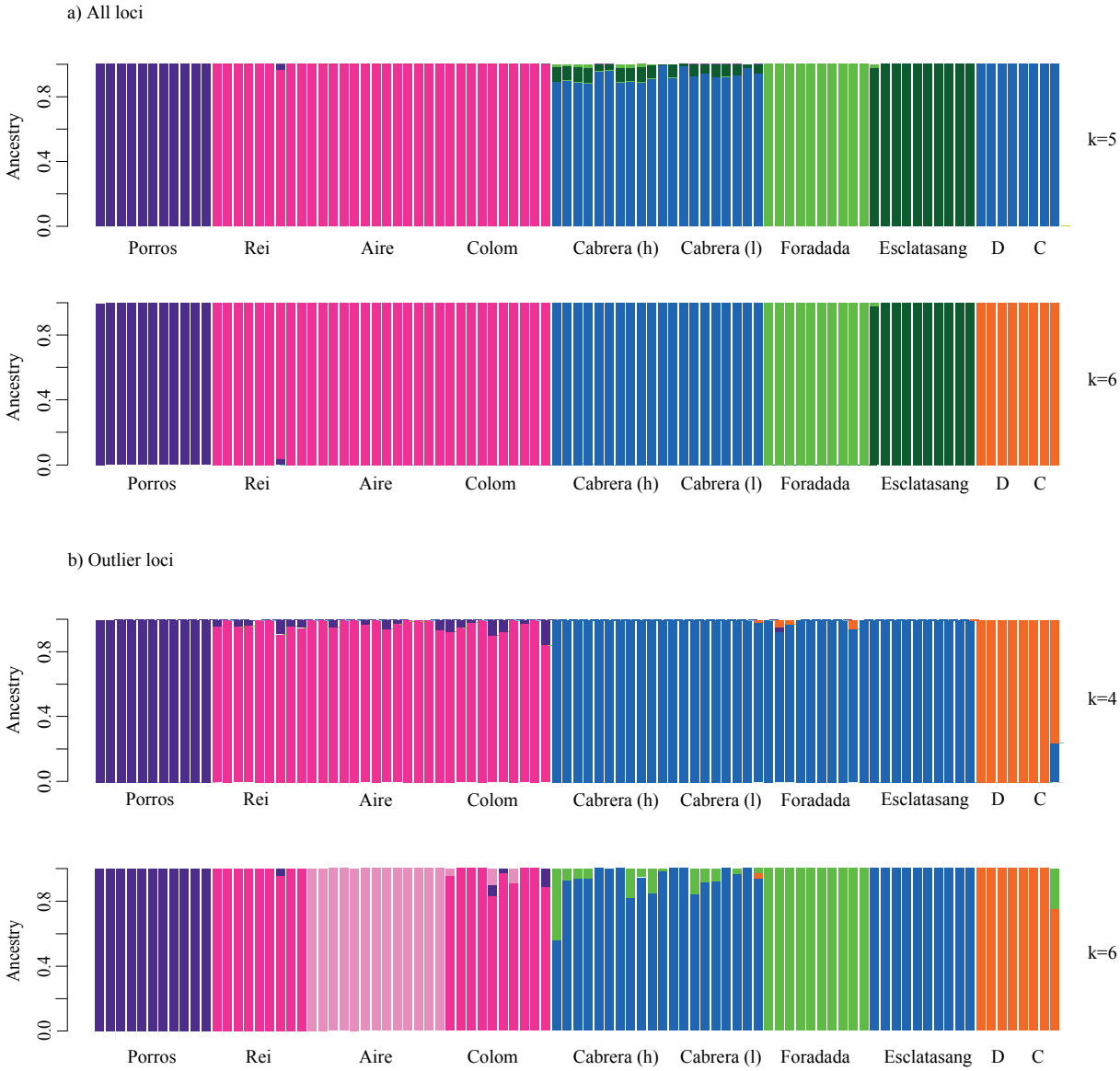


Figure 2. Admixture analysis results with first single SNPs (a) at K=5 and K=6, and only outlier loci (b) at K=4 and K=6. Abbreviations: h, harbour; l, lighthouse; D, Dragonera; C, Colomer.

The best-supported values of K in the Admixture analysis were K=5 (CV=0.372) or K=6 (CV=0.388) for first single SNPs dataset. The divergent position of Porros, Foradada and Esclatasang islands was corroborated by these results; Dragonera and Colomer grouped with Cabrera main island with K=5 or formed an independent group with K=6 (Figure 2). When only outlier SNPs were used, Admixture analyses corroborated the separation into three geographic groups (Menorca, Mallorca and Cabrera), with the exception of Porros islet, when K was set to four (CV=0.288). When K=6 (CV=0.294), Porros, Aire and Foradada were revealed as independent groups (Figure 2). The patterns of differentiation observed in the previous analysis match with the population structure obtained with DAPC analyses. The k-means clustering algorithm, included in DAPC analyses using all SNPs, revealed lowest BIC values for 10 (637.3) clusters. Cross-validation showed that use of the first 15 PCs (55.3 % of variance) provided higher assignment rates (99.5 %) and the lowest root mean squared error (RMSE) (0.016), justifying the use of this subset of PCs in the analysis. The first PC (51.2 % of variance) separated all populations into two major groups: Menorcan populations and all the remaining populations from Mallorca+Cabrera.

All lizard populations grouped by island (Cabrera main island and Dragonera) or islets (Porros, Aire, Foradada, Esclatasang and Colomer), except for Rei and Colom islands in Menorca that grouped together. Ten clusters were also favored when analyses were carried out using only SNPs that were candidates for selection, and variance was best explained by 25 PCs (90.2 % of variance). In this case, the first PC (91.4 %) also showed a separation between Menorca and Mallorca+Cabrera populations. The populations grouped geographically (Menorca, Mallorca and Cabrera), except for Porros islet which continued showing a divergent position (Supplementary Figure 2). The clear separation between Menorcan populations and all the remaining populations from Mallorca+Cabrera was reinforced using both all and only SNPs under selection. Neighbor-Joining (NJ) tree based on F_{ST} distances ratified the results found with previous analyses (Figure 3).

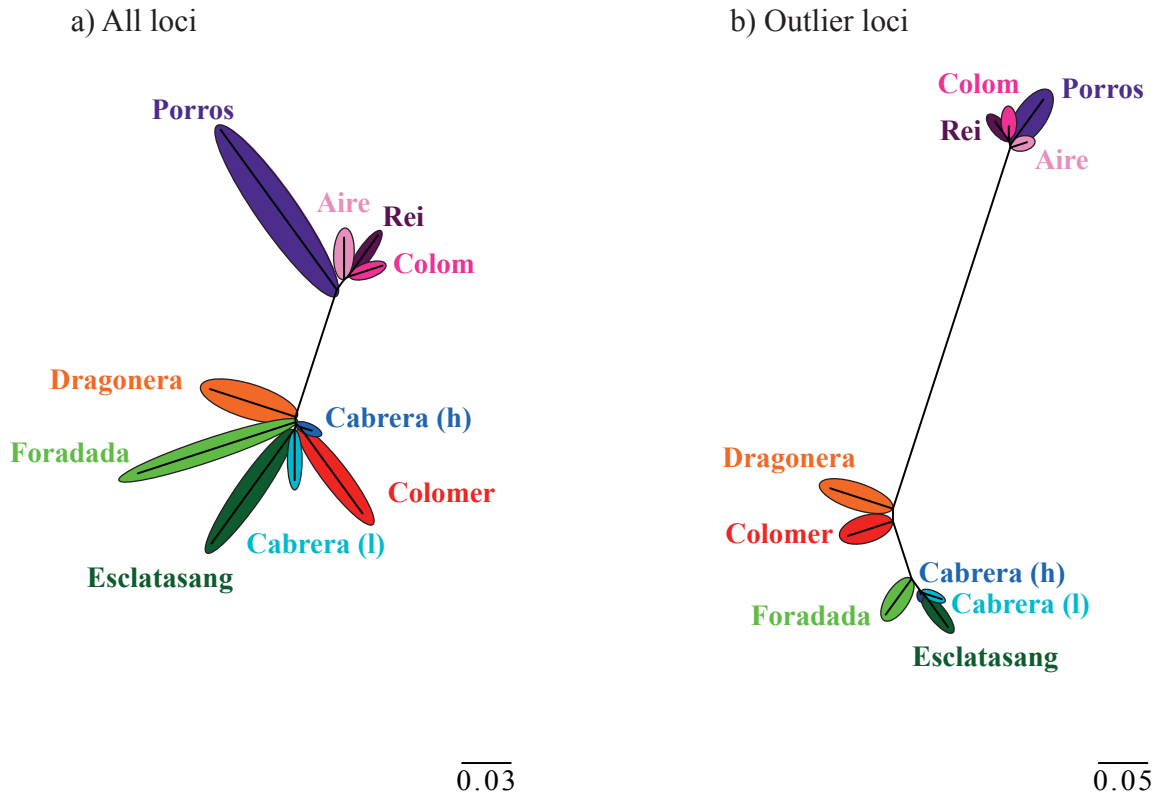


Figure 3. NJ tree based on F_{ST} distances based on first single SNPs (a) and only outlier SNPs (b).

Test of selection

A total of 1,355 candidate sites for selection from 80,091 RAD tags were determined by Bayescan under a prior of 1:100 for selected:neutral sites. This increased to 2,884 sites when a ratio of 1:10 was used, and decreased to 732 sites when the prior ratio was 1:1000. Taking into account the number of SNPs analysed, a true ratio exists between 1:10 and 1:100; thereby, our use of a 1:100 prior provides quite conservative results. After filtering, a total of 141 of the 184 RAD sites that contained outlying SNPs produced hits on BLASTn and hits with <30% query coverage were discarded (Table 1).

Table 1. Gene ID, molecular and biological function of the 1,355 outliers SNPs obtained by Bayescan analysis and the posterior filters. References of studies related with specific biological functions are included.

Gene	Molecular function	Biological function	References
ACACB, ACSBG1	Fatty acid metabolism	Lipid metabolism, hibernation	(Jin et al., 2018; Secor & Carey, 2011)
ADAM2, ADAM9, ADAMTS17	Metalloendopeptidase activity, collagen binding	Fertility, tail regeneration	(Bahudhanapati et al., 2015; Alibardi, 2020)
ADCY1, ADCY2	Adenylate cyclase activity	Inflammatory response, circadian rhythm, axonogenesis	(Tosini et al., 2012)
ANK1, ANKRD13A	Ankyrin	Transcriptional factors, cell regulators, cytoskeletal, ion transporters and signal transducers	(Voronin & Kiseleva, 2007)
CACNA1G, CAMK1D	Calcium signaling pathway	Sperm storage	(Yang et al., 2020a)
CNKSR2	Protein kinase binding	Signal transduction	(The UniProt Consortium, 2017)
COL5A3, COLGALT1	Collagen activity	Skin development	(Geng et al., 2015)
FGFR1	Fibroblast growth factor	Tail regeneration	(Subramaniam et al., 2018; Pillai et al., 2013)
GPC1, GPC4	Growth factor binding	Adipocyte differentiation	(Schoettl et al., 2018)
HS6ST2	Sulfotransferase activity	Muscle cell differentiation	(Wang et al., 2019a)
ITPR2	Calcium signaling pathway	Egg shell quality, muscle contraction, response to hypoxia	(Sun et al., 2015; Ng et al., 2015; Qu et al., 2013)
MAP2, MAP7D3	Microtubule binding	Axonogenesis, neuronal development, sex determination	(Fischer et al., 1987; Singchat et al., 2018)
MYO18B, MYO7B	Motor activity	Sensory perception, actin organization	(The UniProt Consortium, 2017)
OLFM2	Olfactomedin	Muscle cell differentiation	(The UniProt Consortium, 2017)
PBX3	Transcription factor	Embryonic development	(Tosches et al., 2018)
PCDH17, PCDH7	Calcium signaling pathway	Cell adhesion	(The UniProt Consortium, 2017)
TACC1	Hormone receptor	Cell proliferation	(The UniProt Consortium, 2017)
WNT10A	Wnt signaling pathway	Tail regeneration, epidermis morphogenesis	(Vitulo et al., 2017)
ZNF516, ZNF711	Transcription factor	Adipose tissue development, thermogenesis	(Carobbio et al., 2019)

Environmental association analysis

The RDA analysis based on all SNPs indicated that variation due to environmental variables (24.5%) was lower than unexplained variance (75.5%) (Figure 4). However, when the analysis was based only on outlier SNPs (1,355), environmental variables explained most of the variation (63.3%). A total of 58 association loci were found, most of which were related to human pressure (53.4%) and predation (36.2%). Some of these associated SNPs have been found to be related with locomotory and feeding behavior (NEGR1, GRM1), perception of pain (GRM1), lipid metabolism (GDPD2) or ion transport (FHL1, FTH1, SLC9A6), microtubule formation (CLIP1), myoblast differentiation (MBNL3) (The UniProt Consortium, 2017), embryonic development (INTS6L) (Kapp et al., 2013), pH regulation (SLC9A6), toxin transport (DNAJC17), cell adhesion (ESAM, NEGR1), hormone regulation (TG, NCOA1), brain development, and cognition (SHROOM4) (The UniProt Consortiumm 2017).

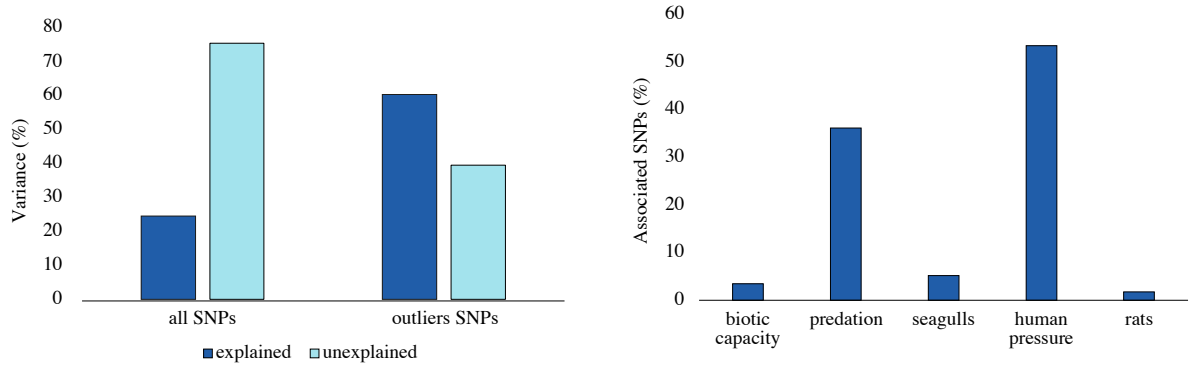


Figure 4. Explained variation due to ecological variables computed in RDA analysis based on first single SNPs (72,846) and outlier SNPs (1,355) is indicated on the left graphic. The percentage of associated SNPs for the retained variables after RDA analysis based on SNPs under selection is indicated on the right graphic.

Discussion

The RADseq methodology has been applied in other squamate studies, enabling the increase of knowledge regarding the processes related to genetic divergence and the identification of genomic regions of interest. The total number of SNPs obtained in this study agree with the SNP density found in other paired-end RADseq works on squamates, with high levels of diversity (Brown et al., 2016; Jin & Brown, 2019). Previous mtDNA analysis of *P. lilfordi* population history pointed out the clear divergence of Menorca and Mallorca clades (Terrasa et al., 2009a) dated around 2.6 Ma (Brown et al., 2008). Colomer population occupied an unclear position, as it grouped with populations from south of Mallorca and north of Cabrera, despite their geographical isolation in north of Mallorca (Terrasa et al., 2009a). Population structure analysis based on both total SNPs (one per RAD tag) (Yang et al., 2020b) and SNPs putatively under selection revealed a clear genetic structure among populations of *P. lilfordi*, validating the already found high divergence between Menorca and Mallorca+Cabrera populations (Terrasa et al., 2009a; Brown et al., 2008). Genome-wide structure analysis based on outlier SNPs revealed that Colomer islet population is genetically closer to Mallorca populations than to Cabrera forms. This finding clarifies the results found in previous mtDNA studies and supports the hypothesis pointed out by (Terrasa et al., 2009a) that the Colomer could be a relict population representative of the ancient populations that once inhabited Mallorca.

The populations with the smallest population size (Porros, Foradada and Esclatasang) showed a divergent position in all genetic structure analyses based on neutral information carried out in this study and furthermore presented the highest F_{ST} values and the greatest number of private alleles, which reinforces their significant divergence from other populations and the effect of genetic drift due to geographical and genetic isolation (Brown et al., 2016; Li et al., 2019). High isolation added to the small size of some of these populations would lead to an expected decrease in genetic diversity and high values of inbreeding (Hedrick & Kalinowski, 2000; Willi et al., 2006; Perrier et al., 2017; Sovic et al., 2019), but although nucleotide diversity values were low (Cao et al., 2020), the inbreeding values were not less than 10% (Lowe & Allendorf, 2010; Ralls et al., 2018), indicating no clear evidence of an inbreeding effect in the *P. lilfordi* populations considered in this study.

It is very interesting to highlight the evidence of adaptive divergence among lizard populations based on F_{ST} outlier test. In this study, almost 2% of total SNPs were candidates to be under selection. These loci turned out to be related to adaptations like tail regeneration, reproduction, lipid metabolism, or circadian rhythm, among other functions. In lizards, other studies revealed a link between genetic variation of candidate genes and the geographical distribution of species, patterns of colonization and/or landscapes gradients (Benestan et al., 2016; Campbell-Staton et al., 2016; Rodríguez et al., 2017b; Prates et al., 2018). In our study, environmental variables appear to be an important driver of divergence between lizard populations, according to the genomic patterns obtained using RDA analysis. The highest number of associated SNPs resulting from the RDA analysis were associated with levels of predation and human pressure in the lizard habitat. These associated SNPs were involved in diverse functions highlighting those related to feeding and locomotory behavior. The influence of the remaining predictors included in the analysis, such as the biotic capacity of islands, the presence of rats, or the existence of breeding colonies of gulls, are negligible. Some behavioral and physiological differences between populations can be related to differences in predation and human pressures, as in the case of escape behavior in lizard populations with or without terrestrial predators. In the Balearic lizard, there is a significant effect of predation pressure on flight initiation distance, distance fled, or hiding time (Cooper et al., 2009, 2010; Cooper & Pérez-Mellado, 2010, 2012).

Overall, our results reveal that both neutral processes, associated with isolation and small population size, and selective factors, related to environmental patterns (specifically human pressure and level of predation) have played a role in shaping divergence between Balearic lizard populations.

Methods

Sample collection, DNA extraction, library preparation, and sequencing

Tissue samples were collected from 94 lizards (*P. lilfordi*) from 10 different sampling locations across the Balearic archipelago (Figure 1 and Table 2). Populations were selected to cover the diversity of surfaces, orography, plant cover, presence of terrestrial predator and human pressure, as well as different population sizes and different mtDNA clades (Table 2). Total genomic DNA was extracted from each tissue sample using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's standard protocol with a specific RNase copurification step. DNA was quantified using the Thermo Fisher Scientific Qubit 3.0 Fluorometer (ThermoFisher Scientific) and quality evaluated using agarose gel and Nanovue Plus Spectrophotometer (GE Healthcare, UK Limited). Paired-end ddRADseq libraries were prepared and sequenced by Floragenex (Eugene, Oregon, USA), following Peterson et al. (2012) and Truong et al. (2012) protocols. Full details are provided in Supplementary Methods.

Data processing and variant calling

Stacks v2.4 (Catchen et al., 2013) pipelines were used to process the sequence reads and call SNPs for each individual. First a demultiplexing and quality filtering step was carried out using *process_radtags* with the default parameters. Clean reads were used to perform a de novo RAD assembly using the *denovo_map.pl* pipeline. The percentage of missing genotypes for each individual was calculated using the *--missing-indv* in VCFtools v0.1.15 (Danecek et al., 2011) and three individuals with more than 79% of missing data were removed. SNPs present in RAD tags found in at least 80% (R) of individuals (Supplementary Figure 3) and with a minimum allele frequency (MAF) of 0.05 were selected and exported into a VCF file using *populations* pipeline. One single SNP per RAD tag was called using *populations* to reduce the effects of linkage disequilibrium. The SNPs dataset was exported to Bayescan (Foll & Gaggiotti, 2008) and to Admixture v1.3.0 (Alexander et al., 2009) format. See Supplementary Methods.

Population structure

Several analyses were used to characterize population structure of island lizard populations based on both entire genome information (first single SNPs, 72,846 SNPs) and only on outlier loci (1,355 SNPs). First, two different programs, Stacks v4.2 (Catchen et al., 2013) and *hierfstat* R package (Goudet & Jombart, 2015), were used to obtain overall levels of genetic variability among different lizard populations. Secondly, population structure was examined with Admixture v1.3.0 program (Alexander et al., 2009) based on both datasets, for K=2 to K=10 co-ancestry clusters. Third, patterns of genetic divergence on both datasets were analyzed using two approaches. Discriminant Analysis of Principal Components (DAPC) performed using the R package *adegenet* (Jombart & Ahmed, 2011) to obtain an overall representation of the divergence between populations and NJ trees were inferred using MEGA7 (Kumar et al., 2016) based on pairwise F_{ST} distances. More information is provided in Supplementary Methods.

Table 2. Characteristics and environmental variables of the studied populations. Abbreviations: N; number of samples used for every population, S; island surface in hectares, predation indexes: absence of terrestrial predators=0; one occasional predators in the island=1; one regular predator was or is present in the island=2; two regular predators present in the island=3, human pressure: uninhabited island and very difficult landing=0; sporadic human presence and easy landing=1; regular human presence and easy access=2; permanent human presence with constructions=3; present and past human presence=4.

Population		N	Population size	S (ha)	Biotic capacity	Vascular plants	Predation	Human pressure	Rats	Seagulls
Menorca	Aire	12	77,500	29.8	6.10	94	0	2	no	yes
	Colom	10	58,107	51.14	7.62	267	1	3	yes	yes
	Porros	10	54	0.05	-2.66	32	0	1	no	no
	Rei	10	1,845	4.08	4.08	204	2	4	yes	no
Cabrera	Cabrera (harbour)	12	534,888	1,137.24	12.18	486	3	3	yes	yes
	Cabrera (lighthouse)	10	5,171	10.6	7.16	486	3	2	yes	yes
	Esclatasang	11	714	0.42	2.69	23	0	0	no	yes
	Foradada	10	1,356	1.61	3.77	19	0	1	no	yes
Mallorca	Dragonera	4	132,875	267.81	11.29	300	0	2	yes	yes
	Colomer	4	10,017	3.05	5.74	8	0	0	no	yes

Test of selection and environmental association analysis

A test of selection was carried out to explore the role of divergent selection on *P. lilfordi* population structure. Bayescan (Foll & Gaggiotti, 2008) was used to identify candidate loci under selection based on an F_{ST} outlier approach.

In the examination of local adaptation to heterogeneous landscapes, the analysis of genome-environment association (GEA) is a central tool (Frichot et al., 2013; Rellstab et al., 2015). Climatic variables were not used as environmental predictors because the Balearic lizard inhabits a reduced geographical range (Pérez-Cembranos et al., 2020). Six environmental traits were considered: biotic capacity, number of vascular plants species, predation pressure, human pressure, and presence/absence of rats and gulls. All these traits are related to natural resources on the islands and disturbing factors to lizards' survival and were selected because showed clear differences among the populations studied. Partial redundancy analysis (RDA) was used as a GEA method to identify adaptive loci based on associations between genetic data and environmental predictors (Forester et al., 2018). See Supplementary Methods.

Ethical statement

All tail tips samples used in this study were obtained in accordance with Ethical Guidelines of the Universities of Balearic Islands and Salamanca, particularly, following the Bioethics Committee Guidelines of the University of Salamanca. The Ethical Committee from the University of Salamanca publishes general Guidelines concerning the experimental protocols with laboratory animals. These general Guidelines for laboratory animals can be read in www.usal.es. According to these Guidelines, only the requirements applicable to our study were implemented, simply because we do not perform any experiment with lizards in captivity. Concerning the field protocol of capture, handling and release of lizards at the site of capture few minutes after the obtention of tail tips, we received the approval from the Nature Conservation Agency (Conselleria de Medi Ambient) of the Government of Balearic Island (permits: CEP 02/2018 and CEP 10/2016 to V.P.-M. and A. P.-C.), that is the administration that has to approve this protocol.

Genomic signatures of drift and selection driven by predation and human pressure in an insular lizard

Supplementary Material

Supplementary Methods, Supplementary Tables

Supplementary Methods

Sample collection, DNA extraction, library preparation, and sequencing

Lizards were caught by careful noosing and 1 cm of tail tip was removed and stored in DNAgard® or in ethanol 100%. All individuals were released at sites of capture after sample collection. The number of individuals sampled in each location ranging between 4 and 12. Populations were selected from 43 known *P. lilfordi* extant subspecies (Pérez-Cembranos et al., 2020) to cover the diversity present in the different lizard populations (Table 2). For each individual, 1 µg of genomic DNA, extracted as indicated in Methods, was sent to Floragenex (Eugene, Oregon, USA) for ddRADseq library construction, following the protocols described in Peterson et al. (2012) and Truong et al. (2012), with modifications. Briefly, 100-500 ng total genomic DNA was double digested using *PstI* (CTGCAG) and *MseI* (TTAA). After digestion, sequence identifier barcodes and sequence adapters were added, and PCR was performed. A 3' primer that selects for fragments which have a "TC" in front of the *MseI* site was used to decrease complexity. Paired-end sequencing with read length of 101 bp was conducted using one lane of the Illumina HiSeq 4000 at the University of Oregon Genomics and Cell Characterization Core Facility.

Data processing and variant calling

Raw read sequences quality was checked using FastQC v0.10.1 (Andrews et al., 2016) and then processed using the *denovo_map.pl* pipeline in Stacks v2.4 (Catchen et al., 2013). First, reads were demultiplexed and filtered using *process_radtags* pipeline. Low quality score (Phred score was set to 10), uncalled nucleotides, and unidentifiable barcode or restriction site were discarded. Clean reads were used to perform de novo RAD assembly using the *denovo_map.pl* pipeline. RAD tags within individuals were aligned using *ustacks* with parameters m (minimum depth of coverage required to form a stack) and M (maximum nucleotide mismatch allowed to merge two stacks) set to three. RAD tags were combined into a catalogue using *cstacks* allowing up to three mismatches between loci (n). Matches of individual RAD tags to the catalogue were searched using *sstacks*. The percentage of missing genotypes for each individual was calculated using the *--missing-indv* in VCFtools v0.1.15 (Danecek et al., 2011) and three individuals with more than 79% of missing data were removed. SNPs present in RAD tags found in at least 80% (R) of individuals and with a minimum allele frequency (MAF) of 0.05 were selected and exported into a VCF file using *populations*. One single SNP per RAD tag was called using *populations* to reduce the effects of linkage disequilibrium. Parameter selection for de novo pipeline was based on the r80 optimization approach (Paris et al., 2017). Optimization tests were run on a subset of 10 individuals (one per population). Different values of (M) and (n) (1 to 6) were tested, with (m) fixed as 3 and R as 0.80. The effects of M and n were evaluated on the number of polymorphic loci obtained with population in Stacks (Supplementary Figure 3). The SNPs dataset was exported to Bayescan (Foll & Gaggiotti, 2008) format using PGDSpider v2.1.1.5 (Lischer & Excoffier, 2012) and to Admixture v1.3.0 (Alexander et al., 2009) format using PLINK v1.90 (Chang et al., 2015).

Population structure

Two different programs were used to obtain indices of population-specific genetic variability based on the entire genome dataset. Stacks v4.2 (Catchen et al., 2013) software was used to calculate the number of private alleles, nucleotide diversity (π), observed (Ho) and expected heterozygosity (He), and inbreeding coefficient (F_{IS}) for all positions and for variant positions. Genome-wide estimates of divergence (F_{ST}) were calculated between all populations applying a p-value correction ($p<0.001$) to exclude insignificant F_{ST} measures. The *hierfstat* R package (Goudet & Jombart, 2015) was used to obtain allelic richness (Ar) Ho, He, F_{IS} and F_{ST} using the *basic.stats* function and bootstrap confidence intervals (1,000 bootstrap replicates). Confidence intervals for population specific F_{IS} and pairwise F_{ST} were determined using the *boot.ppfis* and *boot.ppfst* functions, respectively. Pairwise F_{ST} following Weir & Cockerham (1984) was calculated using the *genet.dist* function for both datasets (first single SNPs and only outlier SNPs). Heatmaps based on F_{ST} were created using *ggplot2* in R (Wickham et al., 2016).

Admixture v1.3.0 program (Alexander et al., 2009) was used to estimate population structure based on first single SNPs and only on outlier loci, assuming different numbers of co-ancestry clusters (K) ranging from 2 to 10. Cross-validation (CV) was set to 10-fold to compare different number of K, in which lower CV value indicates the most likely number of clusters.

Patterns of genetic divergence were analyzed using two different approaches based on the entire dataset and only on SNPs under selection. A Discriminant Analysis of Principal Components (DAPC) was performed using the R package adegenet (Jombart & Ahmed, 2011) to obtain an overall representation of the divergence between populations. In the first step of this analysis, a Principal Components Analysis (PCA) is applied to examine differentiation between individuals regardless of population origin. In the second step, the Principal Components (PCs) that provide suitable predictive power, as assessed by cross-validation, are input into a Discriminant Function Analysis (DFA). Individuals were grouped by population for the DFA, which maximizes among-group relative to within-group variation. Neighbor-Joining (NJ) trees were inferred using Mega 7 (Kumar et al., 2016) based on pairwise F_{ST} distances for both datasets.

Test of selection

Bayescan (Foll & Gaggiotti, 2008) was used to identify candidate loci under selection based on an F_{ST} outlier approach. The method implements a reversible jump MCMC approach that can move between a selection model, containing a population-specific component and a locus-specific component, and a model with just a population-specific component (no selection). The posterior probability of selection at a locus is determined by the proportion of MCMC samples that include the model with the locus-specific component. The prior odds specified for the ratio of neutral:selected sites were set at 1:100, reflecting that neutral sites might be around 100 times more probable than sites under selection (Lotterhos & Whitlock, 2014). This prior can have considerable influence on the number of sites detected, so runs were also carried out with 1:10 and 1:1000 proportions. The MCMC characteristics of the analysis were: 20 pilot runs of 5,000 steps, 5,000 iterations with 50,000 discarded as burn-in, and a sampling interval of 10. Outliers were identified from the results using the R code supplied with Bayescan and significance was determined under a false discovery rate of 5%. We searched the GO biological process and molecular functions using UniProtKB database (The UniProt Consortium, 2017) for outliers with high F_{ST} (> 0.80) and log10(PO) equal to 1000 that blasted to genes in the *Podarcis* genome (NCBI:txid42163).

Environmental predictors

Biotic capacity, number of vascular plants species, predation pressure, human pressure, and presence/absence of rats and gulls were considered as environmental variables for the genome-environment association analysis. The environmental variables used in this study were selected because they showed clear differences among the populations studied, such as the presence of potential competitors and predators, as well as orographic and landscape traits that can be considered proxy of habitat heterogeneity and resource availability.

As an indicator of orography and habitat diversity, for each island we recorded its maximum altitude to calculate the so-called index D of "biotic capacity" (Cheylan, 1992; Parlanti et al., 1988), with the formula: D= log(S x a), where S is the surface of the island (in hectares) and a is its maximum altitude (in meters). The number of vascular plants was employed as an indicator of resource availability in each population. For this variable, data were obtained from several sources (Bibiloni et al., 1993; Pérez-Mellado et al., 2008; unpublished lists and personal observations). The remaining environmental predictors were codified as categorical variables, as performed in a previous study (Pérez-Mellado et al., 2008) (Table 2). Four levels of predation pressure were considered: 0= absence of terrestrial predators; 1= one occasional predator present on the island: there is only one case, Colom Island, where the Ladder snake, *Zamenis scalaris*, an introduced species that very rarely predates on lizards, is present (Pleguezuelos, 1998); 2= at least one regular predator on lizards was or is present on the island: this is the case of Rei Island, where domestic cats, *Felis catus* were present for centuries, when the military hospital was active (Pérez-Mellado & Pérez-Cembranos, 2012); 3= two regular predators on lizards present on the island, such as Cabrera Island, where two carnivorous mammals are still present: the feral cat, *Felis catus* and the Genet, *Genetta genetta*, both of which are important predators of lizards (Pérez-Cembranos et al., 2020). The Yellow-legged gull (*Larus michahellis*) hardly ever captures lizards (Pérez-Mellado et al., 2014) and cannot be considered a lizard predator. Human pressure was codified in four levels: 0= uninhabited island, absence of human visits and/or very difficult landing. This is the case of Colomer Island in Mallorca and Esclatasang islet in Southern Cabrera, both are protected areas with very difficult landing; 1=sporadic human presence and easy landing; 2= regular human presence and easy access; 3= permanent human presence with constructions; and 4= present and past human presence. We included two additional variables for the presence of other vertebrate species that can act, potentially, as competitors of lizards for trophic resources: *Rattus rattus* and two breeding gulls, *Larus michahellis* and *Ichtyaetus audouinii* (Pérez-Mellado et al., 2008 and personal observations).

Lizard densities were estimated during the spring of 2018, employing the line transect method (Thompson et al., 1998) with the R package *unmarked*. This package fits hierarchical models of animal occurrence and abundance to data collected on species subject to imperfect detection (Fiske & Chandler, 2011). Then, lizard population sizes were estimated as the total number of lizards present in each population with that density (lizards/hectare) and the surface covered with vascular plants, estimated from the satellite view of Google maps and personal observations.

Environmental association analysis

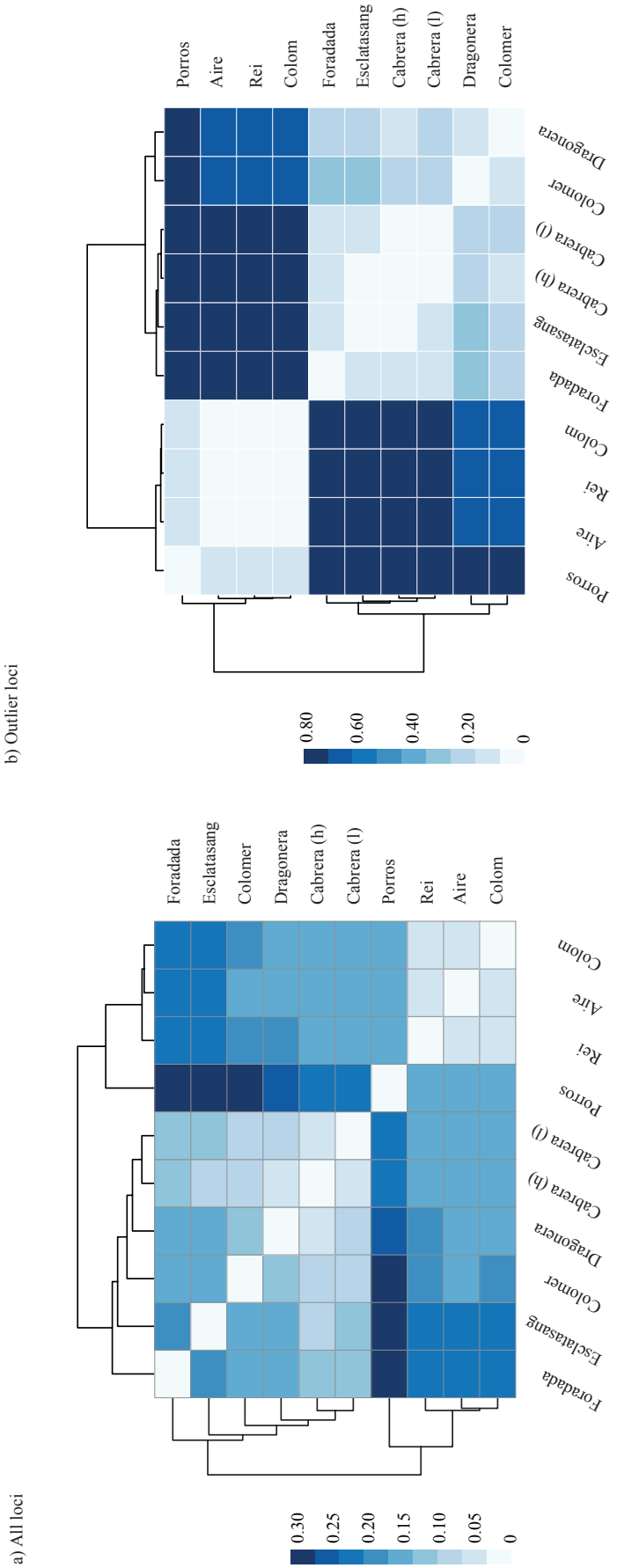
RDA is a multivariate constrained ordination method that can be used to explain differences in population structure using a combination between linear regression and principal component analysis (Borcard et al., 2011). Constrained ordinations explain the relative contribution of the chosen environmental variables in the genetic structure (Legendre & Fortín, 2010). In this study, RDA was performed using *vegan* R package (Oksanen et al., 2017) based on the entire genome data and using the subset of outlier identified by Bayescan.

To estimate the proportion of genetic variation in lizard populations explained by divergent selection, RDA was run including environmental variables (Table 2). RDA analysis was performed imputing missing genotypes on the SNP loci dataset by replacing them with the most common genotype across all individuals. For determination of best model, we applied a forward stepwise selection process with permutation of 999 and a=0.01 using *ordiR2step* function, maximizing adjusted R^2 at every step (Oksanen et al., 2017). To avoid collinearity and redundancy information, retained variables with a variance inflation factor (VIF) over 10 were excluded (Borcard et al., 2011). Finally, RDA analysis was carried out with five environmental variables (predation, human pressure, biotic capacity and presence of seagulls and rats) to obtain the percentage of associated SNPs related to each variable. The UniProtKB database (The UniProt Consortium, 2017) was used to obtain GO biological process and molecular functions of associated SNPs with the highest percentage.

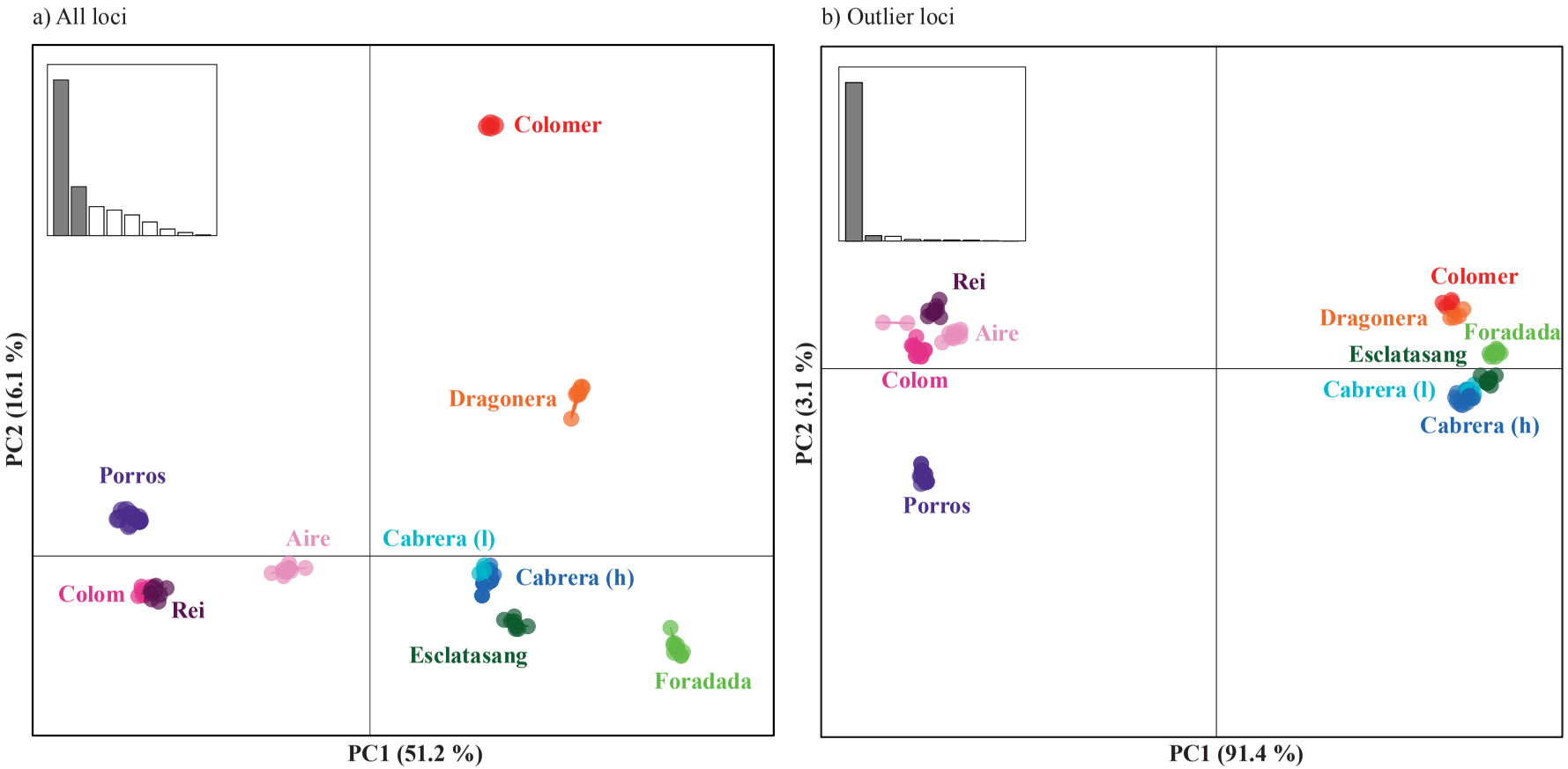
Supplementary Tables

Supplementary Table 1. Values of genetic diversity parameters for 72,846 loci including nucleotide diversity (π), allelic richness (Ar), observed (Ho) and expected (He) heterozygosity and inbreeding coefficient (F_{IS}), considering only variant positions and considering all positions in all *P. lilfordi* populations calculated using *populations* in Stacks and *hierfstat* in R.

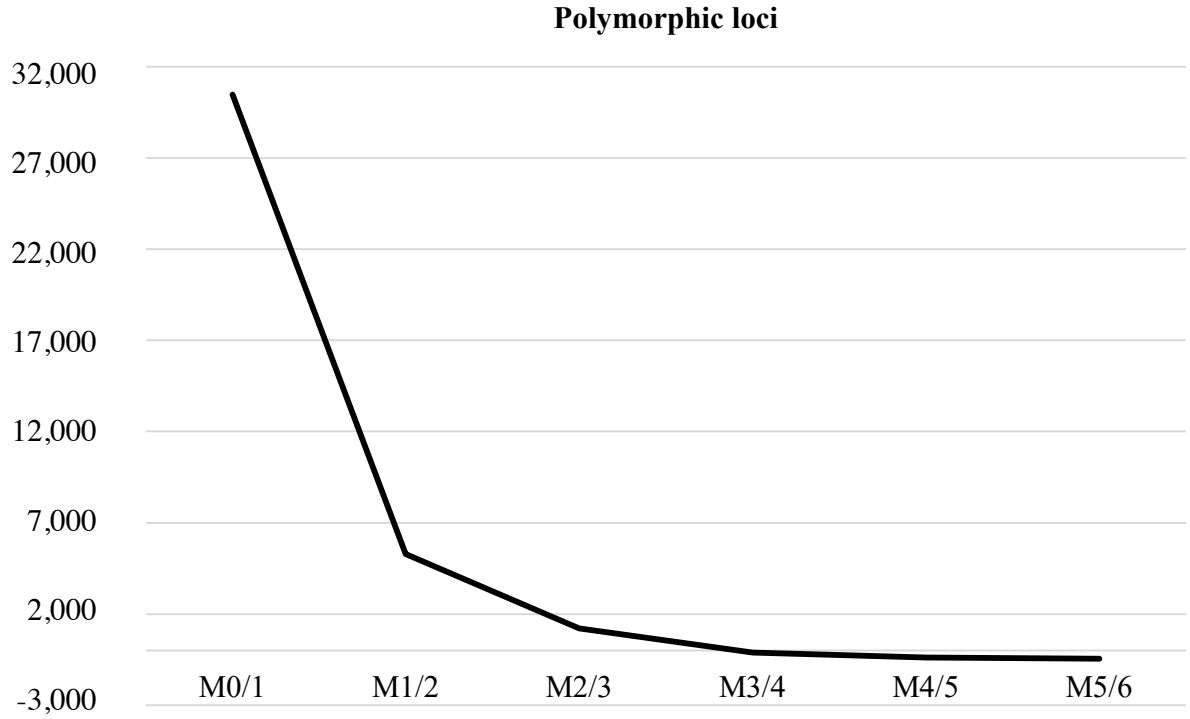
	Population	Private alleles	π			H_o			H_e			F_{IS}		
			Ar	Variant	All	Hierfstats	Variant	All	Hierfstat	Variant	All	Hierfstat	Variant	All
Menorca	Aire	52	1.1661	0.1661	0.0009	0.1534	0.1534	0.0009	0.1586	0.1586	0.0009	0.0328	0.0339	0.0002
	Colom	6	1.1681	0.1681	0.0010	0.1523	0.1523	0.0009	0.1586	0.1590	0.0009	0.0395	0.0405	0.0002
	Porros	945	1.1183	0.1200	0.0007	0.1282	0.1284	0.0007	0.1256	0.1141	0.0006	-0.0203	-0.0175	-0.0001
	Rei	20	1.1637	0.1638	0.0009	0.1575	0.1575	0.0009	0.1598	0.1548	0.0009	0.0147	0.0168	0.0001
Cabrera	Cabrera(harbour)	19	1.1818	0.1818	0.0010	0.1619	0.1619	0.0009	0.1717	0.1737	0.0010	0.0569	0.0573	0.0003
	Cabrera (lighthouse)	3	1.1767	0.1767	0.0010	0.1603	0.1603	0.0009	0.1668	0.1646	0.0009	0.0390	0.0410	0.0002
	Esclatasang	475	1.1655	0.1660	0.0009	0.1562	0.1563	0.0009	0.1596	0.1568	0.0009	0.0216	0.0233	0.0001
	Foradada	746	1.1524	0.1533	0.0009	0.1421	0.1422	0.0008	0.1457	0.1444	0.0008	0.0247	0.0263	0.0001
Mallorca	Dragonera	4	1.1254	0.1269	0.0007	0.1064	0.1066	0.0006	0.1104	0.1092	0.0006	0.0365	0.0405	0.0002
	Colomer	1	1.1219	0.1232	0.0007	0.1114	0.1115	0.0006	0.1133	0.1066	0.0006	0.0172	0.0229	0.0001



Supplementary Figure 1. Heatmaps based on genetic distances (F_{ST} values) among studied populations for first single SNPs (a) and for outlier SNPs (b).

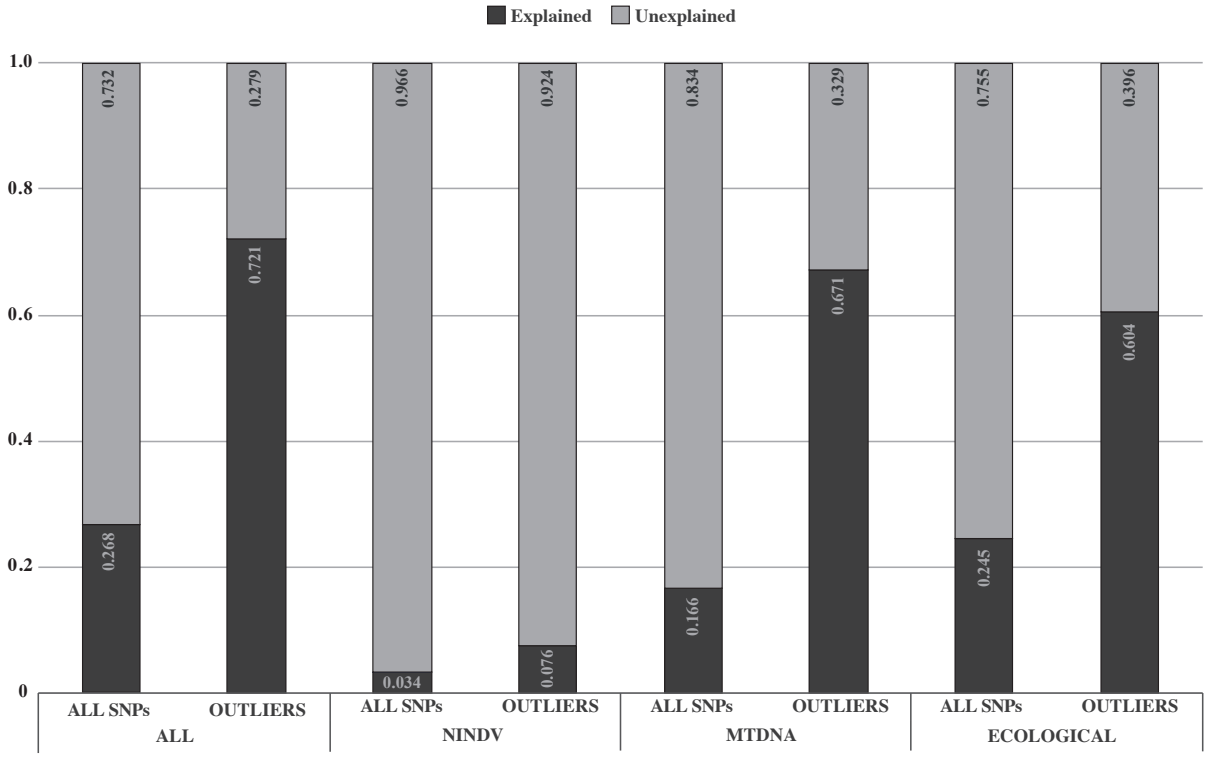


Supplementary Figure 2. DAPC analysis results with first single SNPs (a) and only outlier loci (b).



Supplementary Figure 3. Plot of the number of new polymorphic loci added for each iteration of M=n for a subsample of our database using r80 method (Paris et al., 2017).

Additional information not included in the publication



Additional Figure 1. Percentages of explained and unexplained variation du to different variables: All variables (ALL), number of individuals (NIND), mtDNA divergence (MTDNA) and ecological variables (ECOLOGICAL) computed in RDA analysis based on first single SNPs (72,846) (ALL SNPS) and outlier SNPs (1,355).

Morphological and genetic diversity of the Balearic lizard, *Podarcis lilfordi* (Günther, 1874). It is relevant for its conservation?

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Biosketch

Our research team come from two countries and three different universities. For the last 20 year we have carried out research into the phylogeography, evolution, ecology and behavioural ecology of insular lizards from Western Mediterranean basin. Our approach is multidisciplinary, as we address the study of insular populations of reptiles using molecular techniques, morphological analyses and the study of the most relevant ecological and behavioural traits. We are especially interested in the adaptive features that could explain the survival of insular lizards in ecosystems that provide extreme environmental conditions and in which they appear to survive the pressure from human occupation that began 5,000 years ago.

Running title: Diversity and conservation of Balearic lizards

Abstract

Aims: To characterise the genetic and morphological diversification of the endangered Balearic lizard *Podarcis lilfordi* and to assess the relevance of this diversity, and how it is described, to conservation measures.

Location: This study covers all the populations of the Balearic lizard, *Podarcis lilfordi*, present in its range of distribution at coastal islets of Menorca, Mallorca and Cabrera Archipelago.

Methods: We analysed genetic and morphological variation across the 43 known extant populations of the Balearic lizard, using mitochondrial and nuclear markers. We examined morphometric and scalation characters using, in some cases, phylogenetically independent contrasts. We also incorporated the study of dorsal coloration and dorsal colour pattern including the analysis of melanism in several populations.

Results: We detected clear genetic divergence between Menorcan populations and populations from Mallorca and Cabrera, in both nuclear and mtDNA markers, but genetic divergence is relatively low among different insular populations within these groups. In contrast, morphological divergence was substantial both between Menorcan and remaining populations and within these groups. Morphological traits, such as dorsal coloration, body size and the number and size of scales, seemed to be linked with differences in climatic conditions between populations. In addition, some traits, as melanism, showed a strong phylogenetic signal.

Main conclusions: The morphological and genetic diversity of the Balearic lizard is incongruent with the subspecies described in the classical taxonomic literature. Moreover, current populations not only differ in some genetic and morphological features, but also in several ecological and ethological characteristics, in many cases unique to one population. Based on our results, we propose abandoning the use of subspecies to describe the extraordinary morphological diversity of the Balearic lizard and its replacement with the concept of Evolutionarily Significant Units (ESU). ESUs are particularly suitable to describe and recognize such diversity and, especially, to ensure the continuity of the evolutionary process.

Key words

Podarcis, lilfordi, morphology, scalation, phylogeny, Lacertidae, Conservation, Balearic Islands

1 Introduction

For a variety of taxa, islands make a very important contribution to biodiversity, out of proportion to their land area in comparison with continents. Island contribution to global diversity is mainly in terms of endemic forms, instead species diversity (Whitakker & Fernández-Palacios, 2007). This is the case of Balearic Islands, with only three species of autochthonous terrestrial vertebrates, one midwife toad and two lacertid lizards, all of them endemic to Balearic Islands.

According to IUCN assessment, the Balearic lizard, *Podarcis lilfordi* (Günther, 1874), is an Endangered species (Pérez-Mellado & Martínez-Solano, 2009) of Mediterranean wall lizard endemic from the Balearic Islands (Western Mediterranean, Spain). *Podarcis lilfordi* is a member of the first post-Messinian faunal assemblage reported from Menorca Island (Bover et al., 2008; Bover et al., 2014). During the Holocene, around 2,000 years ago, the species became extinct on the main islands of Mallorca and Menorca, probably as a consequence of the introduction of terrestrial predators by Romans (Pérez-Mellado, 2009 and references therein). It now survives only on the small islets found around the coasts of Menorca and Mallorca, together with Cabrera archipelago and a small introduced population in Colonia Sant Jordi, in Southern Mallorca (Figure 1).

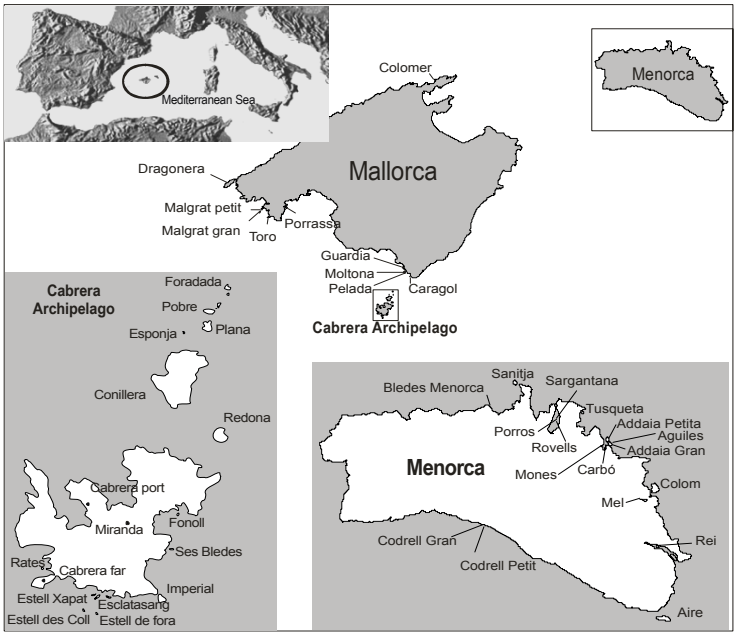


Figure 1. The distribution of major clades of *Podarcis lilfordi* in Western Balearic Islands (Ibiza Island is not represented in the map). We showed all known populations of the Balearic lizard in Menorca, Mallorca and Cabrera Archipelago (see Table 1 for a list of described subspecies and their distribution).

Phylogeographic studies indicate that the Balearic lizard separated from its sister species, *Podarcis pityusensis* (Boscá, 1883), after the refilling of the Mediterranean basin, at the end of the Messinian Salinity Crisis (Brown et al., 2008; Terrasa et al., 2009a and b). Menorcan and Mallorcan populations appear to have diverged at the beginning of the Quaternary period, 2.6 Ma (Brown et al., 2008). Sea levels were higher during the Late Pliocene than during the present day (Emig & Geistdoerfer, 2004), precluding a land connection between Mallorca and Menorca. The genetic separation of lizards from Mallorca, including Cabrera archipelago, and Menorca was maintained during later periods of the Pleistocene, despite potential for secondary contact that could have taken place during eustatic Pleistocene sea level changes (Emig & Geistodoerfer, 2004). Consequently, during more than 2.5 my, the reciprocally monophyletic clades of (Mallorca, Cabrera), and Menorca evolved with little or no introgression. Another major divergence occurred around 2 Ma in Western Mallorca and the rest of populations around Mallorca Island (Brown et al., 2008).

In addition to these major divergences, within the groups of Mallorca and Menorca there has been a subsequent differentiation of isolated populations forming one of the most surprising arrays of allopatric populations. Thus, the Balearic lizard was recognized as a polytypic species (Huxley, 1940 in Mayr, 1963). Taxonomic studies to provide formal recognition of this variation began with Von Bedriaga who defined three infraspecific taxa (Bedriaga, 1879). During the first third of the 20th century, several subspecies of the Balearic lizard were described in a few years by Müller and Eisentraut (Müller, 1927; Eisentraut, 1928a, 1928b; Wetsttein, 1937). The remaining subspecies were described during the second half of the 20th century (Hartmann, 1953; Salvador, 1979, 1980; Pérez-Mellado & Salvador, 1988) (Table 1).

Table 1. Known populations of the Balearic lizard and described subspecies (see Figure 1 for locations of populations under study). We include subspecific assignation for each population and sample sizes (N) of gene fragments studied. In the case of Mel, Colonia de Sant Jordi, Porrasa, Aguiles and Mones there is no subspecific assignation. Pitiusic Islands were include as outgroup indicating in Formentera Island the location of studied samples.

Location	Population	Subspecies	MtDNA (N)	COI (N)
Mallorca	Colomer	*P. l. colomi* (Salvador, 1979)	4	2
	Dragonera	*P. l. gigliolii* (Bedriaga, 1879)	4	2
	Malgrat Petit	*P. l. hartmanni* (Wetsttein, 1937)	2	2
	Malgrat Gran	*P. l. hartmanni* (Wetsttein, 1937)	2	2
	Toro	*P. l. toronis* (Hartmann, 1953)	3	1
	Caragol	*P. l. jordansi* (Müller, 1927)	2	2
	Guardia	*P. l. jordansi* (Müller, 1927)	2	2
	Moltona	*P. l. jordansi* (Müller, 1927)	2	2
	Pelada	*P. l. jordansi* (Müller, 1927)	2	2
	Colonia de Sant Jordi	Undescribed		2
	Porrassa	Introduced	2	2
Menorca	Aire	*P. l. lilfordi* (Günther, 1874)	2	2
	Addaia Gran	*P. l. addayae* (Eisentraut, 1928)	5	2
	Addaia Petita	*P. l. addayae* (Eisentraut, 1928)	3	2
	Colom	*P. l. brauni* (Müller, 1927)	3	2
	Bledes Menorca	*P. l. sargantanae* (Eisentraut, 1928)	2	2
	Codrell Gran	*P. l. codrellensis* (Pérez-Mellado & Salvador, 1988)	2	2
	Codrell Petit	*P. l. codrellensis* (Pérez-Mellado & Salvador, 1988)	3	2
	Carbó	*P. l. carbonerae* (Pérez-Mellado & Salvador, 1988)	3	2
	Mel	Undescribed	4	2
	Porros	*P. l. porrosicola* (Pérez-Mellado & Salvador, 1988)	2	2
	Rovells	*P. l. sargantanae* (Eisentraut, 1928)	3	2
	Sargantana	*P. l. sargantanae* (Eisentraut, 1928)	3	2
	Tusqueta	*P. l. sargantanae* (Eisentraut, 1928)	5	2
	Sanitja	*P. l. fenni* (Eisentraut, 1928)	4	2
	Aguiles	Undescribed	1	4
	Mones	Undescribed		2
	Rei	*P. l. balearica* (Bedriaga, 1879)	2	4
Cabrera	Cabrera Far	*P. l. kuligae* (Müller, 1927)	2	2
	Cabrera Port	*P. l. kuligae* (Müller, 1927)	4	2
	Morro d'en Tià	*P. l. kuligae* (Müller, 1927)		2
	Miranda	*P. l. kuligae* (Müller, 1927)	3	2
	Foradada	*P. l. fahrae* (Müller, 1927)	3	2
	Pobre	*P. l. pobrae* (Salvador, 1979)	2	2
	Plana	*P. l. planae* (Müller, 1927)	3	1
	Esponja	*P. l. espongicola* (Salvador, 1979)	3	2
	Redona	*P. l. conejerae* (Müller, 1927)	2	2
	Conillera	*P. l. conejerae* (Müller, 1927)	1	2
	Rates	*P. l. kuligae* (Müller, 1927)	2	2
	Ses Bledes	*P. l. nigerrima* (Salvador, 1979)	4	2
	Fonoll	*P. l. kuligae* (Müller, 1927)	4	1
	Imperial	*P. l. imperialensis* (Salvador, 1979)	3	2
	Estell des Coll	*P. l. estelicola* (Salvador, 1979)	2	2
	Estell de Fora	*P. l. estelicola* (Salvador, 1979)	2	1
	Estell Xapat	*P. l. xapaticola* (Salvador, 1979)	2	2
	Esclatasang	*P. l. xapaticola* (Salvador, 1979)	3	2

Pitiusic Islands (outgroup)	Na Gorra	*P. pityusensis*		1
	Espardell	*P. pityusensis*	1	1
	Espalmador	*P. pityusensis*	1	1
	Caragoler	*P. pityusensis*	1	1
	Alga	*P. pityusensis*	1	1
	Negra Nord	*P. pityusensis*	1	1
	Porcs	*P. pityusensis*		1
	Eivissa	*P. pityusensis*	1	1
	Vedrà	*P. pityusensis*	1	1
	Formentera (Sant Francesc Xavier)	*P. pityusensis*	1	1
	Formentera (Punta Trocadors)	*P. pityusensis*	1	1

However, the validity of this subspecific arrangement is largely debatable, especially if we take into account the genetics of these insular populations (Terrasa et al., 2009a). The construction of taxonomies based on phylogenetic species concept could remove the need for subspecific descriptions (Haig et al., 2006; Terrasa et al., 2009a). An alternative solution would be the use of Evolutionarily Significant Units (ESU), that initially were defined as units that should be reciprocally monophyletic for mtDNA alleles and that show a significant divergence of allele frequencies at nuclear loci (Moritz, 1994). We will explore the arrangement of these ESU in the case of the Balearic lizard and its consistence with the traditional separation of subspecies.

The temporal process of separation during lineage divergence can accumulate genetic, ecological and morphological changes, resulting in a better adaptation to local environmental conditions. The occupation and use of different habitats by lizards would lead to a divergent selection on traits that define several morphological characteristics as body size, body shape, coloration patterns or scalation characters (Hu et al., 2019; Muñoz et al., 2013; Wollenberg et al., 2013). We analyse, within the frame of genetic variability, these morphological characteristics in all extant populations of the Balearic lizard. Nuclear DNA markers are analysed for the first time and we describe the morphometry, scalation and colour patterns of populations. Morphological traits are also related with different climatic conditions at different geographic locations (Mayr, 1963). In Balearic Islands, due to their latitudinal situation, climatic conditions can be different for the two main clades of Mallorca and Menorca populations. Thus, we tested the potential influence of main climatic conditions of Mallorca and Menorca, that is, rainfall and environmental temperatures, on morphological characters of lizards.

Our goal is to answer the following questions: 1. How valid is the current taxonomic arrangement of populations if we consider their genetic and morphological variability? And 2. What is the relationship between the observed morphological diversity and the evolutionary history of populations (phylogeny) or their current climatic conditions.

2 Methods

2.1 Specimens

We studied all known populations (Figure 1) of *P. lilfordi*, although we did not obtain genetic, morphometric or coloration information from all of them. Samples from Cabrera Island were obtained from four sites: Cabrera port (the area around the Cabrera Bay), Cabrera far (the Ansiola lighthouse Peninsula, at the Southwestern corner of the Island), Morro den Tià (another Western peninsula of the island) and Miranda (central part of Cabrera Island, Figure 1). Note that the population studied from the Bay of Palma (Mallorca), at Porrassa Islet, is known to have been introduced, as well as the small population of Colonia de Sant Jordi in Southern Mallorca.

2.2 Climatic characteristics

The aridity of the Balearic Islands was estimated with the I_q index (Sahin, 2012). In this index, the ratio of annual precipitation to annual mean specific humidity (Sh) is employed. The mean specific humidity can be easily computed with mean temperature, relative humidity and local pressure which are the most commonly measured meteorological data (Sahin, 2012). Due to the limited geographic distribution of *P. lilfordi*, we were only able to compare climatic data from three weather stations: the Port of Palma de Mallorca (Palma Puerto, B228, Mallorca Island), Palma de Mallorca International Airport (Palma Aeropuerto, B278, Mallorca Island) and Menorca International Airport (Menorca Aeropuerto, B894, Menorca Island). The two weather stations on Mallorca Island are very close. We employed data from these weather stations freely provided by AEMET Open Data (Agencia Estatal de Meteorología, Spain). Aridity indices and rainfall were calculated with annual data from 1981 to 2018.

2.3 Genetic study

2.3.1 DNA extraction, amplification and sequencing

Total genomic DNA was extracted from tail tips following standard protocols (González et al., 1996). A total of 104 (Table 1) individuals of *P. lilfordi* have been studied for four non-overlapping mtDNA fragments: (i) partial 12S rRNA, (ii) two partial fragments of cytochrome b (CYTB), (iii) partial control region (CR), (iv) two partial subunits of the NADH dehydrogenase gene and associated tRNAs (referred to as ND1, ND2, $tRNA_{Ile}$, $tRNA_{Gln}$, and $tRNA_{Met}$). Primers and amplification conditions are the same as those used in our previous studies of *Podarcis* (Rodriguez et al., 2013a; Terrasa et al., 2009a). Additionally, 92 (Table 1) individuals were sequenced for COI fragment using primers LCO-1490: GGT CAA CAA ATC ATA AAG ATA TTG G and HCO-2198: TAA ACT TCA GGG TGA CCA AAA AAT CA (Folmer et al., 1994). Specimens of *Podarcis pityusensis* were used as outgroup. One nuclear gene was amplified and sequenced for 45 samples: melanocortin 1 receptor gene (MC1R) (720 bp). Primers and amplification conditions are the same as those used in Buades et al. (2013).

Both strands of the PCR products were sequenced and carried out on an automated ABI 3130 sequencer (Applied Biosystems, Foster City, CA, USA) using a BigDye® Terminator v. 3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and edited using CodonCode Aligner software (CodonCode Corporation, Dedham, MA, USA). Nuclear data were phased using the PHASE algorithm (Stephens et al., 2001) within DnaSP v.6 (Rozas et al., 2017). All GenBank accession number are indicated in Table S8 from supplementary material.

2.3.2 Divergence and phylogenetic analyses

Distance-based method (p-distance) based on mitochondrial data (mtDNA and COI fragment) between subspecies were calculated in MEGA 7 (Kumar et al., 2016) to stablish the level of sequences divergence. We used a dataset including 46 *P. lilfordi* populations for genetic analyses (Table 1). Three different datasets were used: 1) concatenated mitochondrial four DNA fragments (12S, CYTB, CR and NADH) providing an alignment of 2,382 bp length, 2) COI fragment alignment with a length of 656 bp and 3) phased nuclear alignment (MC1R) with a length of 720 bp. Sequences were aligned in the MAFFT v7.423 online server (Katoh et al., 2017) using the iterative refinement method (FFT-NS-i).

For the protein-coding genes, alignments were verified by translating nucleotide sequences to amino acids. Best-fit nucleotide substitution models and partitioning scheme were chosen simultaneously using PartitionFinder V1.1.1 (Lanfear et al., 2016) under the Akaike Information Criterion (AIC). The partitioning schemes were defined by hand with the "user" option, with branch lengths of alternative partitions "unlinked" to search for the best-fit scheme, which consisted of one partition for the mtDNA dataset: [HKY+G+I] and 2 partitions for the COI dataset: 1st and 2nd position [HKY+I] and 3rd position [GTR+G].

Bayesian analyses were performed with MrBayes 3.2.1 (Ronquist et al., 2012). The analyses were run for 10^6 generations with sampling frequency every 10^3 generations. Numbers of runs and chains were left as default, two and four, respectively. Sufficient number of generations was confirmed by examining the stationarity of the log likelihood (lnL) values of the sampled trees and the value of average standard deviations of the split frequencies being lower than 0.01. Results were analysed in Tracer v1.6 (Rambaut et al., 2014) to assess convergence and effective sample sizes (ESS) for all parameters. A burn-in of 25% was applied and the phylogenetic trees were visualized and edited using Figtree v1.4.2 (Rambaut, 2012).

The genealogical relationships between the haplotypes of the phased nuclear gene was inferred using the TCS statistical parsimony network approach (Clement et al. 2000) with 95% connection limit implemented in the program PopART 1.7 (http://popart.otago.ac.nz) (Clement et al., 2002; Leigh & Bryant, 2015).

2.4 Morphological analysis

The morphology of 5,755 specimens (3,455 males and 2,300 females) was studied (Tables S1 to S6). Six body dimensions, as well as body mass (Weight), were included in this study: snout-vent length (SVL), intact tail length (TL), pileus length (PL), head width (HW), head height (HH) and hindleg length (HLL). All measurements were made with a digital calliper to the nearest 0.01 mm, with the exception of SVL and intact tail length, which were measured with a steel rule to the nearest 1 mm. Weight was obtained with a spring scale Pesola®. Six scalation characters were studied: gularia, collaria, dorsalia, ventralia, left femoralia, and left fourth digit lamellae (see Pérez-Mellado & Gosá, 1988 for methodological details of body measurements and scalation counts). Obviously, not all characters were recorded from all individuals and samples sizes are very variable, according with availability of adult lizards from each population (see Tables S1 to S6). All body measurements were done by the same researcher (VPM).

Morphometric and scalation characters were compared among the three groups of populations (Mallorca, Cabrera and Menorca) using ANOVA type, Lawley-Hotelling type, Bartlett-Nan-Pillai type and Wilks' Lambda type test statistics, as implemented in the R-package *npmv* (Burchett et al., 2017). These statistics were then used as the basis for permutation or randomization tests. Nonparametric relative effects of each morphometric and scalation character were also tested. These effects give an indication of stochastic superiority, i.e. they measure the probability that a value obtained from one group is larger than a value randomly selected from the whole data set. In agreement with default settings of npmv, we employed the results of Wilks' Lambda in pairwise comparisons, and because N>30, we used F approximation (Burchett et al., 2017).

In addition, we employed a non-metric multidimensional scaling (NMDS) to establish morphological divergence, separately for adult females and males, among 43 populations of *P. lilfordi*. The method aims to depict the inherent pattern of a dissimilarity matrix in a geometric picture with a minimum number of dimensions (Clover, 1979). We used the metaMDS function from the *vegan* R-package (Oksanen et al., 2018). This function runs NMDS several times from random starting configurations, compares results, and stops after detecting two similar minimum stress solutions (Oksanen et al., 2018). The goodness-of-fit of the ordination was assessed by the coefficients of determination (R^2) for the linear and non-linear regressions of the NMDS distances on the original ones (Borcard et al., 2011). Finally, we recorded the stress values of NMDS (Zuur et al., 2007). All calculations were done within R environment (R Core Team, 2019).

Dorsal colouration of lizards was analysed with the *colordistance* R-package (Weller, 2019; Weller & Westneat, 2019). This package is an objective comparative tool to colour profiling and comparison of digital images. The standard RGB image analysis cannot provide the wavelength resolution of reflectance spectrophotometry. However, the use of digital images can reflect the visual sensitivities of several species (Losey et al., 2003; Weller & Westneat, 2019).

Our colour analysis was restricted to 30 populations of adult males and 27 populations of adult females of the Balearic lizard from which we had good JPEG dorsal colour images. We employed 2-15 individual images per population. Non-background pixels were binned to read images into the R environment as 3D arrays. We used colour histograms as the binning method in a 5 x 2 x 3 hue-saturation-value (HSV) colour space. HSV is the most suitable method when different digital cameras or variable light conditions were employed to obtain images (Weller & Westneat, 2019). For calculating the distance between one binned image and another, we employed the earth mover´s distance or Wasserstein metric (EMD) method. In this way, colour histograms from each image or from a group of images (i.e., from all individuals of a given population), are compared with a symmetrical distance matrix that is plotted as its corresponding heatmap. In the heatmap we used default colours ranging from yellow (least similar) to blue (most similar).

2.5 Comparative analysis

We did comparative analyses of morphological traits using the mtDNA tree (Figure 2). Due to the low levels of divergence and consequently the high numbers of polytomies, we were unable to obtain an ultrametric and fully dichotomous tree for most comparative analyses. Thus, we restricted our analysis to the comparison of morphological continuous variables with the *caper* package (Orme et al., 2018), using the method of Pagel (1992) to calculate contrasts at polytomies. We compared in this way all continuous variables of morphometry and scalation using SVL as covariate (Orme et al., 2018). In addition, to study the presence/absence of melanic coloration, we employed the D statistics (Fritz & Purvis, 2010) for binary variables of the *caper* package. In order to standardise the effects of phylogeny size and prevalence, *phylo.d* uses two simulated null models: A model of phylogenetic randomness, where trait values are randomly shuffled relative to the tips of the phylogeny, and the Brownian threshold model, where a continuous trait is evolved along the phylogeny under a Brownian process and then converted to a binary trait using a threshold that reproduces the relative prevalence of the observed trait. D typically varies between 0 and 1. A D value close to zero indicates that the binary trait evolves on a tree following the Brownian model (i.e., strong phylogenetic signal).

3 Results

3.1 Climatic characteristics in the Balearic Islands

We found significantly lower aridity (I_q index) in Menorca ($F_{2,111} = 12.59$, $p= 1.18 \times 10^{-5}$), but no differences between Mallorca port and airport (Tukey test, $p>0.05$; Mallorca port, $\bar{x} = 49.13 \pm 2.0$, n=38, min: 23.25, max: 75.83; Mallorca airport, $\bar{x}= 50.07 \pm 1.8$, n=38, min: 28.15, max: 70.3; Menorca airport, $\bar{x} = 62.68 \pm 2.52$, n=38, min: 32.4, max: 93.2). Average annual rainfall was also significantly higher in Menorca ($F_{2,111} = 12.91$, $p= 9.13 \times 10^{-6}$), but again, there were no differences between Mallorca airport and port (Tukey test, $p>0.05$; Mallorca port, $\bar{x}= 455.2 \pm 19.08$ mm, n=38, min: 201.3, max: 702.2; Mallorca airport, $\bar{x}= 415.78 \pm 15$ mm, n=38, min: 227.2, max: 559.6; Menorca airport, $\bar{x} = 548.03 \pm 21.95$, n=38, min: 272.2, max: 811.7).

3.2 Genetic analysis

We have sequenced four fragments plus COI of mtDNA genome. The major genetic differentiation has been detected between Menorca and Mallorca+Cabrera populations, as can be seen in the Bayesian analysis based on COI fragment (Figure 2) and mtDNA concatenated dataset (Figure 3). The latter shows a better resolution of the cladogenesis of the Balearic lizard and its relationship with subspecific assignation. In all analyses, there is a large amount of polytomies, especially in the case of Menorca clade (Figures 2 and 3). Only populations from Western Mallorca (Dragonera, Malgrat islets and Toro), exhibit a clear monophylia of subspecies (*P. l. gigliolii, P. l. hartmanii* and *P. l. toronis*, respectively, Figure 3). Genetic distances estimated between different subspecies based on concatenated mtDNA fragments (Table 2), shows levels of differentiation range between 0.001 and 0.035, and confirm these conclusions. Genetic distances based on COI fragment (not included) show similar results.

Overall, the TCS network built from phased nuclear gene MC1R (Figure 4) exhibits a low variability (it is necessary to say that a minor number of samples are used). It also shows high gene flow between populations from Mallorca and Cabrera and confirmed the clear separation between Menorca and the remaining populations.

In Porrassa island inhabits an introduced population, it is included in phylogenetic trees and exhibits a high proximity to Cabrera archipelago. Thus, the most probable origin could be Cabrera Island, as in the case of Colonia Sant Jordi lizards (Figure 2).

Table 2. Evolutionary divergence over sequences using p-distances among defined subspecies of *P. lilfordi* based on the four mtDNA fragment

	jordansi	introduced	colomi	fahrae	pobrae	planae	espongicola	conejerae	kuligae	xapaticola	estelicola	imperialensis	nigerrima	gigliolii	hartmanni	toronis	addayae	lilfordi	carbonerae	codrellensis	brauni	porrosicola	balearica	fenni	sargantanae
jordansi																									
introduced	0,005																								
colomi	0,007	0,008																							
fahrae	0,007	0,008	0,006																						
pobrae	0,005	0,001	0,007	0,007																					
planae	0,005	0,005	0,006	0,004	0,004																				
espongicola	0,007	0,008	0,006	0,002	0,007	0,003																			
conejerae	0,005	0,003	0,007	0,006	0,003	0,003	0,005																		
kuligae	0,012	0,012	0,013	0,013	0,012	0,012	0,013	0,012																	
xapaticola	0,014	0,014	0,013	0,013	0,013	0,013	0,013	0,013	0,010																
estelicola	0,014	0,014	0,014	0,014	0,014	0,014	0,013	0,014	0,008	0,006															
imperialensis	0,015	0,016	0,016	0,015	0,016	0,015	0,015	0,015	0,005	0,009	0,006														
nigerrima	0,014	0,015	0,015	0,015	0,015	0,015	0,014	0,015	0,004	0,009	0,006	0,001													
gigliolii	0,023	0,024	0,023	0,023	0,023	0,022	0,022	0,023	0,025	0,024	0,025	0,026	0,026												
hartmanni	0,024	0,025	0,024	0,024	0,024	0,023	0,024	0,024	0,026	0,025	0,026	0,026	0,026	0,004											
toronis	0,024	0,025	0,024	0,024	0,024	0,024	0,024	0,024	0,026	0,025	0,026	0,027	0,026	0,006	0,006										
addayae	0,026	0,027	0,027	0,027	0,027	0,026	0,027	0,026	0,028	0,028	0,029	0,030	0,030	0,032	0,033	0,033									
lilfordi	0,026	0,028	0,028	0,027	0,028	0,027	0,027	0,027	0,029	0,029	0,030	0,031	0,030	0,033	0,034	0,034	0,001								
carbonerae	0,027	0,028	0,028	0,028	0,028	0,027	0,027	0,027	0,029	0,029	0,030	0,031	0,030	0,033	0,034	0,035	0,002	0,003							
codrellensis	0,026	0,027	0,027	0,027	0,027	0,026	0,026	0,026	0,028	0,028	0,028	0,030	0,029	0,032	0,033	0,034	0,001	0,001	0,001						
brauni	0,026	0,027	0,027	0,027	0,027	0,026	0,026	0,026	0,028	0,028	0,029	0,030	0,029	0,032	0,033	0,033	0,001	0,001	0,002	0,000					
porrosicola	0,026	0,028	0,027	0,027	0,028	0,027	0,027	0,027	0,028	0,028	0,029	0,030	0,029	0,033	0,034	0,034	0,001	0,002	0,002	0,000	0,001				
balearica	0,026	0,028	0,028	0,027	0,028	0,027	0,027	0,027	0,029	0,029	0,030	0,031	0,030	0,032	0,033	0,034	0,002	0,001	0,003	0,002	0,002	0,002			
fenni	0,027	0,028	0,028	0,028	0,028	0,027	0,027	0,027	0,029	0,029	0,030	0,031	0,031	0,032	0,033	0,034	0,002	0,002	0,003	0,002	0,002	0,002	0,003		
sargantanae	0,026	0,027	0,027	0,027	0,027	0,026	0,026	0,026	0,028	0,028	0,029	0,030	0,030	0,032	0,033	0,034	0,001	0,001	0,002	0,001	0,001	0,001	0,002	0,002	

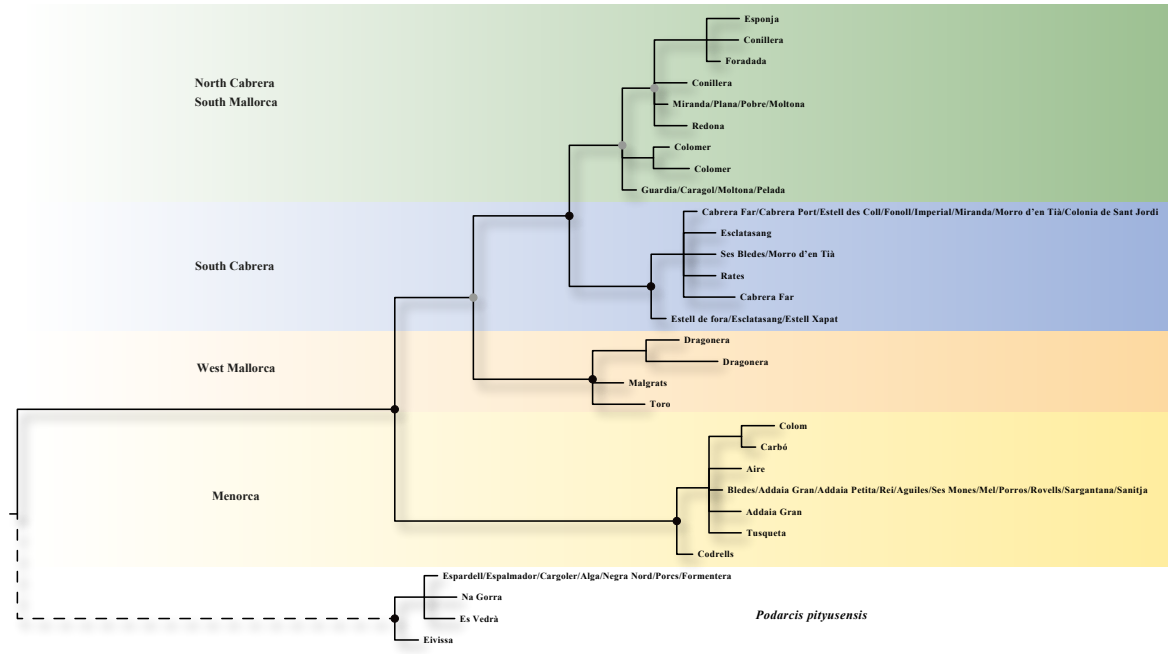


Figure 2. Bayesian analysis based on COI fragment (656 bp). Codrells include Codrell Petit and Codrell Gran and Malgrats included Malgrat Petit and Malgrat Gran. Black dots represent a posterior probability (PP) greater than 90 and grey dots a PP ranged between 80 and 90.

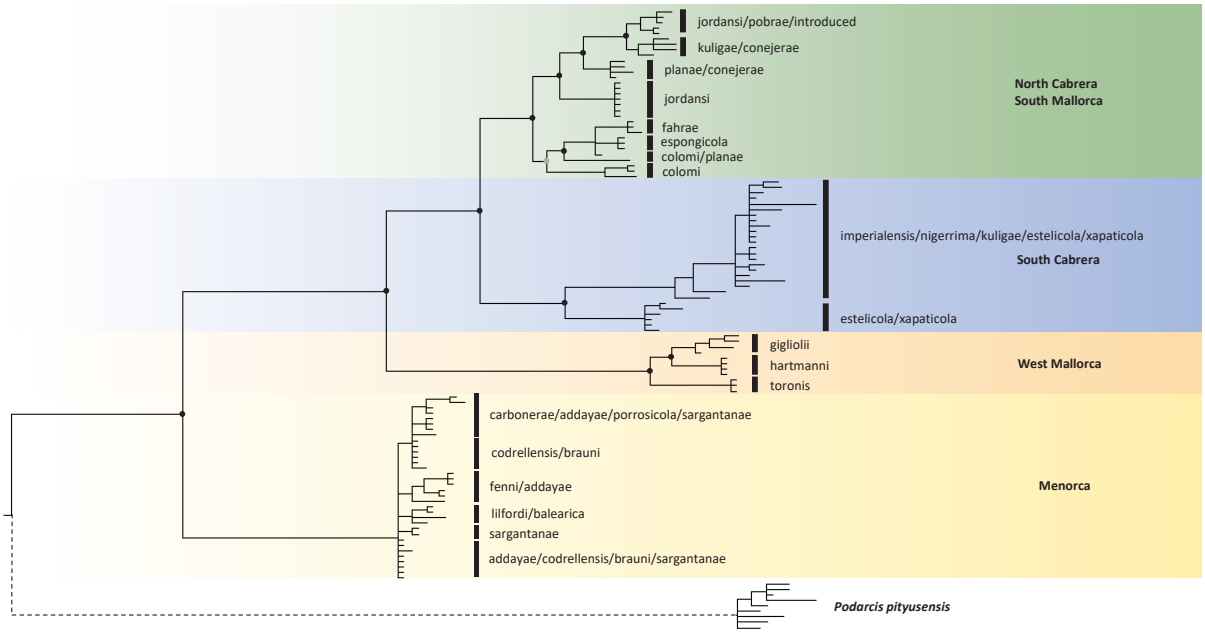


Figure 3. Bayesian analysis based on mtDNA concatenated dataset (2,382 bp). Clades are arranged by described subspecies, excluding "introduced" corresponding to the population of Porrassa islet. Black dots represent a posterior probability (PP) greater than 80 and grey dot a PP smaller than 80.

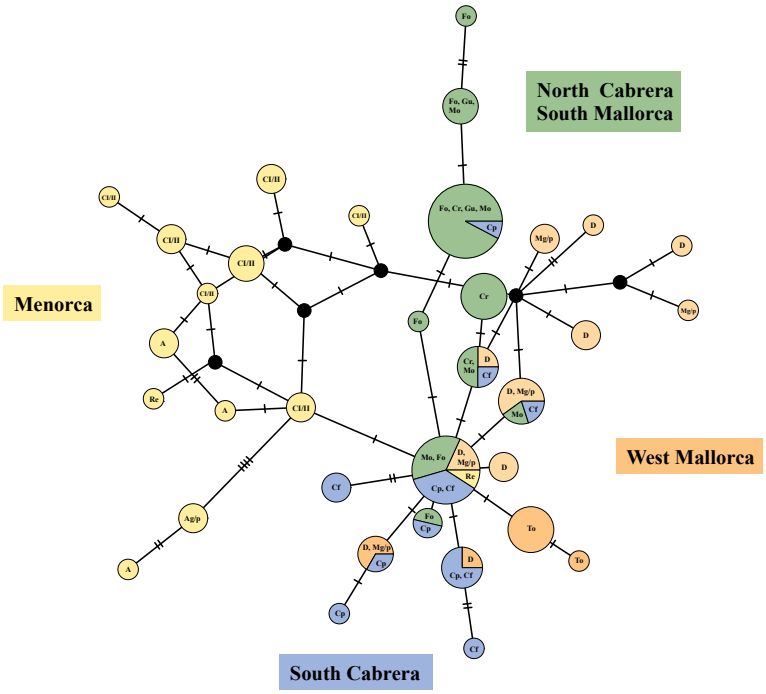


Figure 4. TCS network based on phased nuclear gene MC1R for some populations of the four well-delimited clades: 1) North Cabrera and South Mallorca (green), 2) South Cabrera (blue), 3) West Mallorca (orange) and 4) Menorca (yellow). Every population is represented by its abbreviation (Cr: Colomer, Fo: Foradada, Gu: Guardia, Mo: Moltona, Cf: Cabrera_Far, Cp: Cabrera_Port, To: Toro, D: Dragonera, Mp/g: Malgrat Petit (Mp) and Malgrat Gran (Mg), A: Aire, Re: Rei, CI/II: Codrell Gran (CI) and Codrell Petit (CII) and Ag/p: Addaia Gran and Addaia Petita).

3.3 Morphometry and scalation

With the exception of lamellae ($F_{1,1592}$ = 1.835, p= 0.176), all morphological traits are sexually dimorphic in *P. lilfordi* (Table S7). Thus, all analyses were done separately for adult males and females. Morphology shows a transition from Menorca to Mallorca and Cabrera characterized by increased body size (see Figures 5 and 6 for SVL, and Tables S1 to S6), and a decrease in adjusted head dimensions (Tables S1 to S6), both in adult males and females. Body size (SVL) differs among the three major clades of Mallorca, Menorca and Cabrera (one-way ANOVA, $F_{2, 3452}$ = 35.34, p= 6.39 x 10^{-16}, Menorca, $\bar{X}$ = 68.5 ± 0.1mm, 45-86 mm, n= 2,628; Mallorca, $\bar{X}$= 69.72 ± 0.23 mm, 55-81 mm, n=383; Cabrera, $\bar{X}$ = 70.44 ± 0.23 mm, 57.4-83 mm, n= 444), with larger lizards in Mallorca and Cabrera than in Menorca (Tukey, p= 0.00002 and p<0.001, respectively), but similar sizes between Mallorca and Cabrera (Tukey, p=0.093).

Using phylogenetically independent contrasts, PL, PW, HLL, lamellae and ventralia traits are significantly different among adult males of the populations under study (results in Table 3). PL, HH, HLL, gularia, dorsalia and collaria are different in adult females (Table 4).

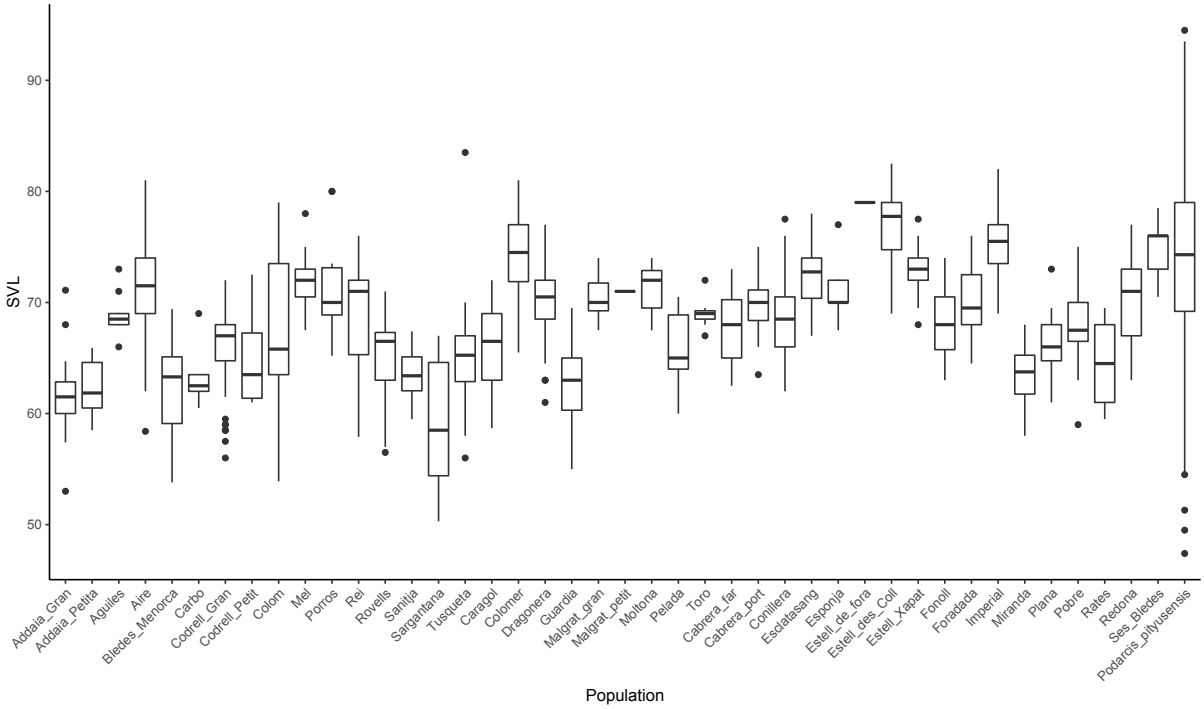


Figure 5. Boxplots of SVL of adult males from Balearic lizard populations ranged by archipelagoes. From the left to the right, Menorca, Mallorca and Cabrera. See the increase of body size from Menorca to Cabrera.

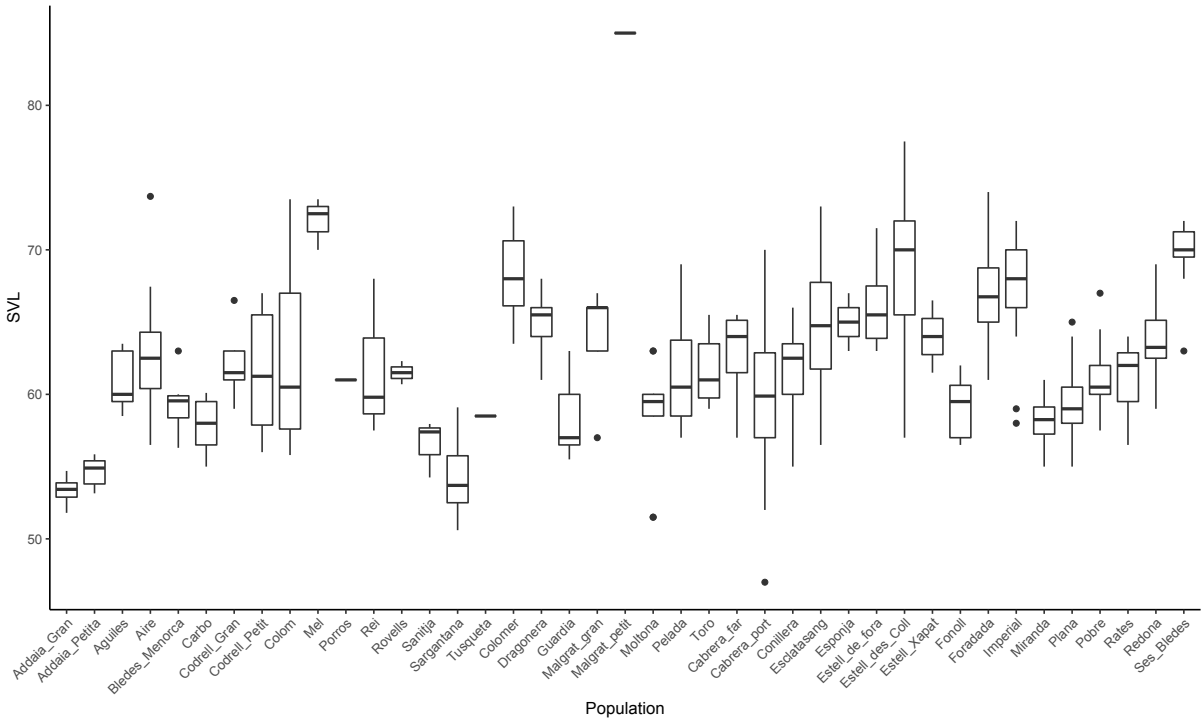


Figure 6. Boxplots of SVL of adult females from Balearic lizard populations ranged by archipelagoes. From the left to the right, Menorca, Mallorca and Cabrera.

Table 3. Results of linear models comparing all Balearic lizard populations, with phylogenetically independent contrasts, each log-transformed morphometric trait, using SVL as covariate. Adult males.

Trait	Model	Adjusted R^2	$F_{1,24}$ - value	p-value
Pileus length	logPL~logSVL	0.3426	14.03	0.001
Head Heigth	logHH~logSVL	0.02081	1.531	0.2279
Pileus width	logPW~logSVL	0.3632	15.26	0.000667
Hindleg length	logHLL~logSVL	0.6372	44.9	$6.246x10^{-7}$
Lamellae	loglam~logSVL	0.1977	7.16	0.01322
Femoralia	logfem~logSVL	0.06596	2.765	0.1093
Gularia	loggul~logSVL	0.09029	3.481	0.07434
Ventralia	logven~logSVL	0.1721	6.196	0.02013
Collaria	logcol~logSVL	0.03918	2.019	0.1682
Dorsalia	logdorsalia~logSVL	-0.01892	0.5358	0.4713

Table 4. Results of linear models comparing all Balearic lizard populations, with phylogenetically independent contrasts, each log-transformed morphometric trait, using SVL as covariate. Adult females.

Trait	Model	Adjusted R^2	$F_{1,24}$-value	p-value
Pileus length	logPL~logSVL	0.1406	5.091	0.03344
Head Heigth	logHH~logSVL	0.5368	29.97	1.296×10^{-5}
Pileus width	logPW~logSVL	0.1021	3.844	0.06163
Hindleg length	logHLL~logSVL	0.153	5.515	0.02743
Lamellae	loglam~logSVL	-0.00801	0.8013	0.3796
Femoralia	logfem~logSVL	0.002392	1.06	0.3135
Gularia	loggul~logSVL	0.1874	6.765	0.01567
Ventralia	logven~logSVL	-0.03289	0.204	0.65555
Collaria	logcol~logSVL	0.1533	5.527	0.02727
Dorsalia	logdorsalia~logSVL	0.3042	11.93	0.002063

Table 5. Mallorca and Cabrera groups. For each test statistic we give its statistical value, numerator (df1) and denominator (df2) degrees of freedom and p- values using both, F approximation and permutation (randomization) methods (see more details in the text).

	Test Statistic	df1	df2	P-value	Permutation Test p-value
ANOVA type test p-value	6.185	6.279	44.7713	0.000	0.002
McKeon approx. for the Lawley Hotelling Test	5.413	26.000	81.5385	0.000	0.000
Muller approx. for the Bartlett-Nanda-Pillai Test	4.314	27.167	102.8553	0.000	0.000
Wilks Lambda	4.878	26.000	98.000	0.000	0.000

Table 6. Nonparametric inference of multivariate samples comparing adult female lizards from Menorca, Mallorca and Cabrera groups. For each test statistic we give its statistical value, numerator (df1) and denominator (df2) degrees of freedom and p- values using both, F approximation and permutation (randomization) methods.

	Test Statistic	df1	df2	P-value	Permutation Test p-value
ANOVA type test p-value	2.101	6.953	46.0228	0.063	0.103
McKeon approx. for the Lawley Hotelling Test	3.961	26.000	81.5385	0.000	0.000
Muller approx. for the Bartlett-Nanda-Pillai Test	3.303	27.788	52.0683	0.000	0.000
Wilks Lambda	3.657	26.000	48.000	0.000	0.000

Table 7. Estimated nonparametric relative effects, showing intergroup differences as probabilities for adult males (see more details in the text).

Group	SVL	Tail	Weight	PL	HH	HW	HLL	LAM	FEM	GUL	DOR	VENT	COLL
Cabrera	0.88542	0.89062	0.96094	0.88281	0.86198	0.85156	0.96615	0.48958	0.79948	0.19531	0.27604	0.44531	0.42188
Mallorca	0.74062	0.84766	0.78750	0.65781	0.24062	0.63203	0.66641	0.47969	0.40547	0.51094	0.50391	0.63984	0.54766
Menorca	0.43015	0.40885	0.41651	0.44654	0.52956	0.45343	0.43995	0.50460	0.50092	0.51578	0.51241	0.47580	0.49525

Table 8. Estimated nonparametric relative effects, showing intergroup differences as probabilities for adult females.

Group	SVL	Tail	Weight	PL	HH	HW	HLL	LAM	FEM	GUL	DOR	VENT	COLL
Cabrera	0.52137	0.80342	0.76496	0.50427	0.45726	0.50000	0.59402	0.65385	0.55556	0.13248	0.27778	0.38034	0.47863
Mallorca	0.71635	0.79487	0.68109	0.64423	0.29808	0.68750	0.67628	0.49679	0.34936	0.46795	0.52724	0.67308	0.52724
Menorca	0.43590	0.38324	0.41987	0.45833	0.56227	0.44643	0.43956	0.48443	0.53709	0.54853	0.51603	0.46337	0.49451

The nonparametric multivariate analysis (ANOVA type test, see above), rejects the hypothesis of equality among male lizards from Cabrera, Mallorca and Menorca (Table 5, $p<0.001$ in all tests). There is an overall significant difference in body dimensions and scalation between Mallorca and Menorca lizards ($p<0.05$) and Cabrera and Menorca lizards ($p<0.05$), but not between Mallorca and Cabrera archipelagos ($p>0.05$). In adult females, we also reject the hypothesis of equality among Cabrera, Mallorca and Menorca (Table 6, $p<0.001$). We find significant differences in body dimensions and scalation between Mallorca and Menorca lizards ($p<0.05$) and Cabrera and Menorca lizards ($p<0.05$), but again, we do not find significant differences between Mallorca and Cabrera ($p>0.05$).

The analysis of relative effects shows higher values in terms of probabilities for all morphometric and scalation characters of male lizards from Cabrera, with lowest values for all characters from male lizards from Menorca (Table 7). Similar tendencies are observed in females (Table 8). In males, higher probabilities correspond to the length of intact tails, body mass, head height and pileus length of lizards from Cabrera. In females, all probabilities of effects of each variable are lower than in males, indicating a closer distance of their morphologies among the three groups of islands. Only tail lengths of Cabrera females showed a value above 0.80 (Table 8).

NMDS analysis of populations of adult males confirms the morphological divergence of lizards from Menorca, situated in the left side of the diagram (Figure 7), with a central overlap of Cabrera Island, Guardia and Caragol from Cabrera and Mallorca clades, respectively, and Aguiles and Aire Island from Menorca group. The right side of the diagram is occupied by lizard populations from Cabrera and Mallorca. The picture is less clear in adult females, with a right position of Mel and Mones populations from Menorca (Figure 8). Thus, NMDS diagrams only partially reflect the geographical arrangement of populations, with a better picture in adult males.

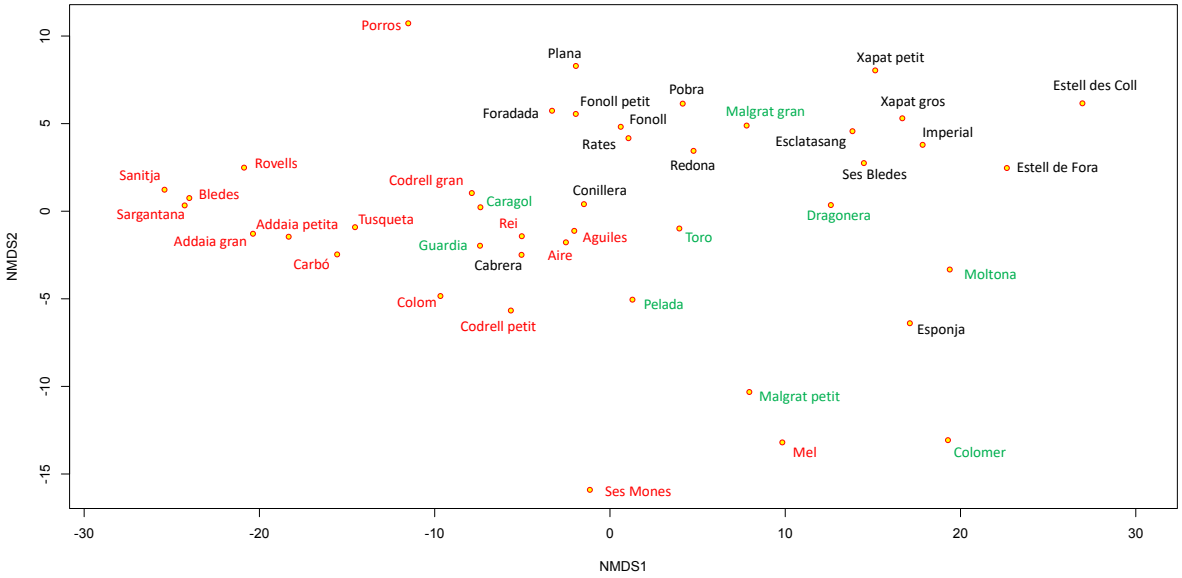


Figure 7. Non-metric multidimensional scaling biplot of a Euclidean dissimilarity matrix of log-transformed morphometric data from 43 populations of adult males of the Balearic lizard. Black: populations from Menorca, Green: populations from Northern Cabrera and Southern Mallorca, Blue: populations from Southern Cabrera and Orange: populations from Western Mallorca. Colours of major clades according to phylogeny of *P. lilfordi* from Figure 3.

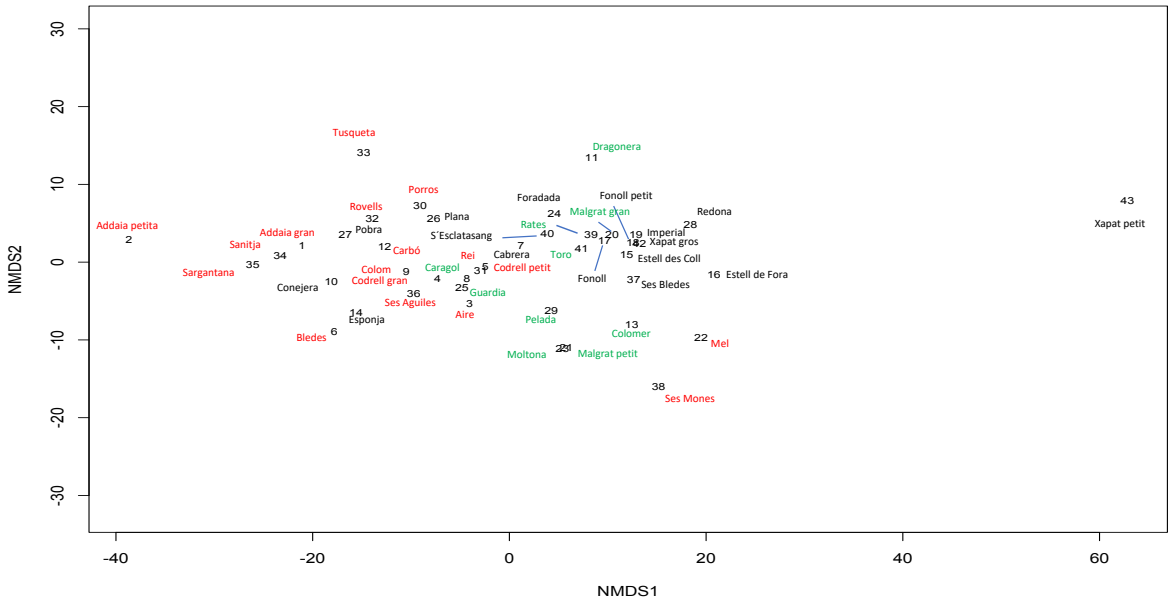


Figure 8. Non-metric multidimensional scaling biplot of a Euclidean dissimilarity matrix of log-transformed morphometric data from 43 populations of adult females of the Balearic lizard. Colours as in Figure 7.

3.4 Patterns of lizard coloration

The comparative analysis of presence/absence of melanism, indicates that this trait has a strong phylogenetic signal (D=0.1267, a value significantly different from a fully random evolution, p<0.001 and with a high probability to be the result of a Brownian evolution, p=0.98). For males (Figure 9) and females (Figure 10), we find clear groups of melanistic and non-melanistic populations. In adult males, non-melanistic lizard from Dragonera and Cabrera, are grouped with some populations from Menorca while the remaining non-melanistic populations of Menorca are grouped together in another clade (Figure 9). Conversely, the only melanistic Menorcan population (Aire) is grouped with the remaining populations of Cabrera and Mallorca. In adult females, the grouping of populations according to dorsal colour is even clearer. A first large group separates all melanistic or almost melanistic females from Menorca (Aire Island), Mallorca and Cabrera, with the remaining non-melanistic females mixed in the second clade (Figure 10 and Supplementary Material).

In lizard populations from Menorca, we find only one (5.88%) or two (11.76%, if we include melanistic individuals from Aguiles Islet) melanistic populations. In populations of Mallorca and Cabrera, melanism is dominant among populations, with 7 of 9 extant native populations (77%) in Mallorca coastal islands and 10 of 14 populations in Cabrera archipelago (71.43%). In fact, in Cabrera Island, the subpopulations of Morro den Tià and Cabrera far, show also a clear tendency for melanism and were grouped by colour analysis together with melanistic populations in the case of adult females (Figure 10), and in a lesser measure in the case of adult males (Figure 9), where there is a group of melanic lizards (Esclatasang, Malgrat Gran and Guardia) and a group of very dark lizards (Cabrera far, Morro den Tià and Toro islets). This result indicates a clear tendency for melanism linked with the isolation in small islets and concomitant demographic effects of that isolation.

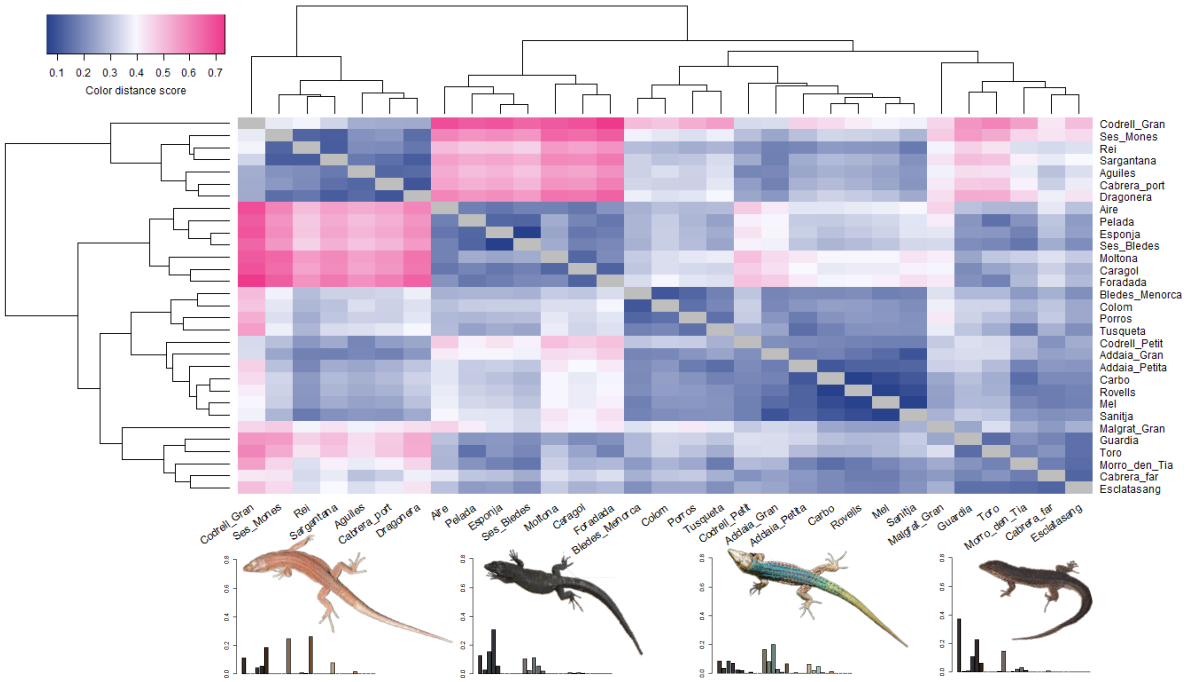


Figure 9. Heatmap of adult males of *P. lilfordi* obtained from dorsal digital images. The dorsal image pixels have been grouped into a 5 x 2 x 3 HSV colour space. Lower values (in blue) indicate greater dorsal colouration similarity of populations and higher values (in pink), a greater distance among them. At the base of the figure we show four histograms and pictures of males from Aguiles, Aire, Porros and Toro populations, as a representation of each of the main clades of the heatmap. The histograms show the percentage of pixels of each of the 5 x 2 x 3 = 30 colour categories employed in the analysis. The first five bars of each histogram correspond to the black colour (see, for example, its dominance in Aire and Toro populations). In turn, bars 6, 11, 16, 21 and 26 correspond to brown colours, from the darkest to the lightest (present, for example, in the case of Aguiles). The rest of intermediate bars of the histograms correspond to green and blue tones, clearly important in males from Porros Islet (see more details in the text). Colours of population's labels as in Figure 7.

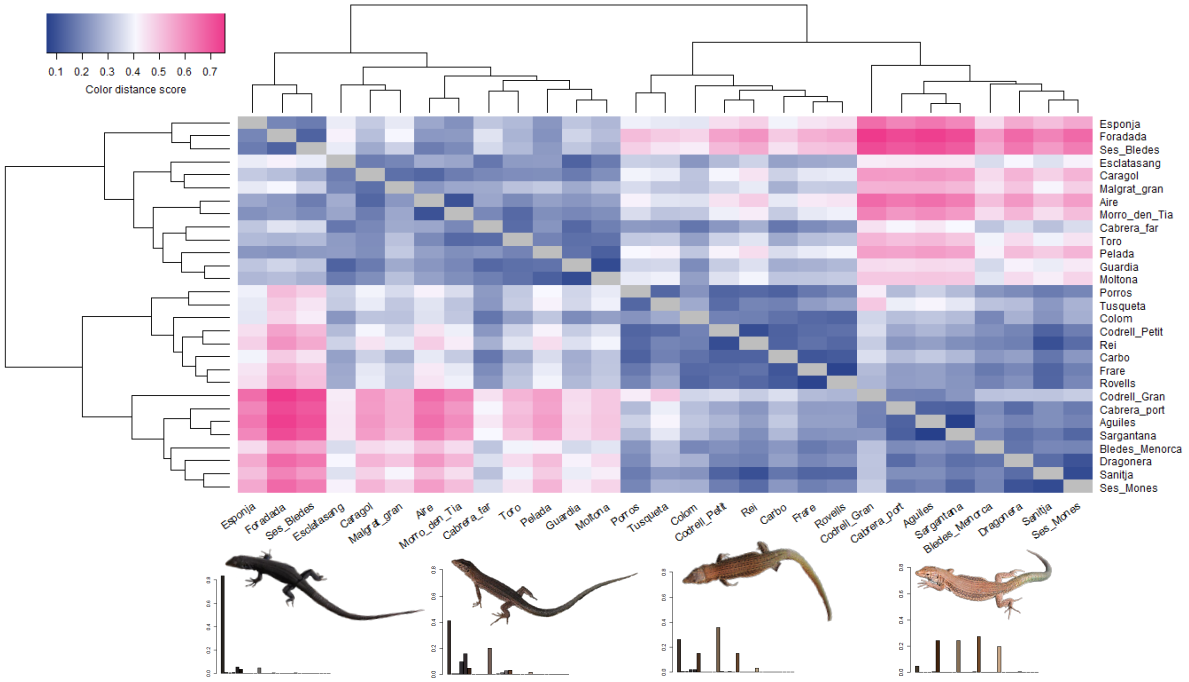


Figure 10. Heatmap of adult females of *P. lilfordi* obtained from dorsal images. As a representation of each of the main clades of the upper heatmap, we include four pictures from Malgrat Gran, Toro, Rovells and Cabrera port populations, with their corresponding histograms. Colours of population's labels as in Figure 7.

4 Discussion

The evolutionary framework of the Balearic lizard

The Balearic lizard was a member of the first post-Messinian faunal assemblage reported from Menorca Island (Bover et al., 2008; Bover et al., 2014). This fauna spread during the Early and Middle Pliocene, including a lizard (*Podarcis* sp., Bailón, 2004) which could be the ancestor of *Podarcis lilfordi*. In Mallorca Island, some remains of a lizard have been found in deposits from the Early Pleistocene to the Holocene and were described as *Podarcis* sp. Some of the additional remains from Middle Pleistocene to Holocene were then ascribed to *Podarcis lilfordi* (Bailón, 2004; Bover et al., 2014). Hence, fossil remains of the Balearic lizard were found from a more recent period in Mallorca than in Menorca. These results are in agreement with the cladogenesis that separated Menorca and Mallorca+Cabrera clades some 2.6 Ma. That is, during the beginning of the Quaternary, at a significantly cooling period (Brown et al., 2008 and references therein). During that period, many cladogenetic events occurred around the world (Paulo et al., 2001; Veith et al., 2003; Guo et al., 2005).

Even if there is a large variability among microinsular populations at the whole range of the geographical distribution of the Balearic lizard, we found significant differences between Menorca and Mallorca and between Menorca and Cabrera populations, while less clear differences are present in morphology between Cabrera and Mallorca. This pattern is in under a significantly more arid climate than those from Menorca, confirming this expectation. However, this is only the present-day situation. The separation of Menorca and Mallorca+Cabrera took place 2.6 Ma, coinciding with the transition from Pliocene to Pleistocene periods (Walker et al., 2018). Since the Late Pliocene/Early Pleistocene, the climatic conditions of Balearic Islands were predominantly humid-Mediterranean (Wagner et al., 2014). Then, during the transition from Late Pleistocene to the Holocene, when took place the final isolation of lizards from Cabrera populations, it was a rising of temperatures, with a warm temperate climate and dry summer/humid winter seasons (Wagner et al., 2014), coinciding with the decrease of the number of dorsal scales in Mallorca and Cabrera populations.

Melanism of the Balearic lizard

Melanistic lizards appeared in several population of the Balearic lizard and our comparative analysis of dorsal colouration grouped all melanistic populations, in spite of their geographic situation. We can speculate that melanistic colouration confers relevant adaptive advantages to its holders, that would explain its appearance in different lineages of the Balearic lizard. It is interesting to note that in the clade of *P. lilfordi* from Menorca, only Aire Island, the oldest coastal islet (Pérez-Mellado, 2009), has a melanistic population, while in supposedly derived populations of Mallorca lizards, melanism has been probably an adaptive response fairly common, both on the coast of Mallorca and in Cabrera archipelago. In fact, our results indicate that melanism is an evolutionary trait with a strong phylogenetic signal.

Taxonomic arrangement of P. lilfordi according to genetic and morphological traits

As expected, if we compare the arrangement of described subspecies of the Balearic lizard with genetic, morphological and colouration results, it is clear a lack of congruence of present-day accepted subspecies (Table 1). Most of the described subspecies are not supported by genetic divergence of clades (Figures 2 and 3), nor by morphometric and colouration traits.

Moreover, in morphology we found striking differences among the three groups of populations, Menorca, Mallorca and Cabrera, but with an arrangement unrelated with described subspecies. In the case of colouration, a trait commonly employed in classical subspecific diagnoses and descriptions, the arrangement matched populations according with dominant dorsal colours, regardless their geographic location or their subspecific membership. However, it is interesting to note that, at least in adult males (Figure 9), there is a clade of non-melanistic populations of Menorca, well separated from a mixture of Mallorca and Cabrera populations, melanistic and non-melanistic, in the right side of the diagram. In females, an isolated clade is formed by the bulk of melanistic populations of Cabrera, Mallorca and Aire Island (Figure 10).

Some cases merit a particular comment. In Menorca, the populations of Codrell Gran and Codrell Petit, described as *P. lilfordi codrellensis*, are very close each other, barely separated by an arm of sea of no more than fifteen meters and widely isolated from the remaining populations of Menorca (Figure 1). However, in the case of males, their morphological distance is very noticeable. It is also the case of males from Malgrat Gran and Malgrat Petit islets, in the southwestern coast of Mallorca. These populations were collectively described as *P. lilfordi hartmanni* and forming, from the genetic viewpoint, a clear monophyletic clade (Figure 3). Two lizard populations from Menorca remain undescribed, from Mones and Mel islets (Table 1). Both are morphologically well separated (Figures 7 and 8), but with a strong genetic similarity to populations apparently unrelated, as in the case of Addaia Gran and Mel (Figures 2 and 3). Some of the described subspecies of *P. lilfordi* are morphologically and genetically differentiated, as those from Dragonera, Malgrat islets and Toro in Mallorca coast (Figures 2 and 3). However, it is not the case of several populations of the Balearic lizard described as different subspecies, as *P. lilfordi lilfordi* from Aire Island and *P. lilfordi balearica* from Rei Island (Terrasa et al., 2009a and this study).

Insular populations of terrestrial vertebrates encounter a physical impediment to gene flow between populations and it is therefore expected that such populations may diverge in isolation (Mayr, 1963). Being frequently smaller in population size, the fixation of neutral genes is likely to take place in a faster way than in continents. However, use of neutral genes might reasonably yield no difference among some populations, even if these populations differ markedly in phenotype. It is useful to estimate the historical patterns of divergence among populations (O´Brien & Mayr, 1991) and population differences had a high conservation utility, as proxies for the sub-structure found within species (Phillimore & Owens, 2006). However, from a cladistic viewpoint, the usefulness of subspecies is a controversial issue (Vinarski, 2015; Wilson & Brown, 1953; Winker, 2010). As classificatory units, subspecies are not useful in comparative studies, because subspecies are groups of populations defined by hypothetical biological relations or geographical distributions, rather than by homology, that is, shared derived characteristics (Ebach & Williams, 2009). In this context, the use of subspecific categories is extremely difficult.

Would it make sense to describe new subspecies for such populations, solely based on morphological differences? From our viewpoint, if that is the question, we should apply the same criteria to describe as different subspecies all the populations separated by morphological differences, without paying attention to genetic differences between them. On the contrary, if we apply strictly genetic criteria, the proximity of numerous populations would force us to invalidate numerous subspecies previously described only with morphological criteria, but unsustainable from a genetic perspective.

The application of Evolutionary Significant Units to Balearic lizards

Solving this dilemma can only be achieved abandoning the very concept of subspecies in the case of the Balearic lizard. As an alternative, we propose the use of Evolutionarily Significant Units (ESU, Moritz, 1994, 2002). In addition to its original genetic definition (Moritz, 1994), an ESU was also defined as a group of organisms substantially reproductively isolated that represent an important component in the evolutionary legacy of the species (Waples, 1995). This is the case of every microinsular population of *P. lilfordi*, physically isolated even from closest islets. An ESU can often corresponds to species or subspecies boundaries in classical taxonomy, but in some cases, it can be extended to isolated populations (Karl & Bowen, 1999).

The ESU has been proposed as a unit of conservation (Vogler & DeSalle, 1994; Moritz, 1994; Waples, 1995). We have to evaluate each population along two axes of diversity which might be described as molecular genetics and adaptative morphology. Adaptive diversity is the diversity of morphological and ecological traits we observe today in different populations of *P. lilfordi*. This diversity represents the raw material for future evolution. Consequently, we have to recognize every isolated population of the Balearic lizard as an ESU (Pérez-Mellado, 2008).

In this study we have only addressed the description and possible origin of some of the morphological features of the Balearic lizard. However, it remains to be elucidated the origin and causality of a myriad of ethological and ecological features that, in one way or another, characterize each population (Pérez-Mellado, 2009 and references therein). This is the task that implies the complete preservation of the microevolutionary process in the islets where the Balearic lizard survives for thousands of years. The potential taxonomic identity of each population is not its most relevant aspect and it is paradoxical that it is the main aspect that has attracted the attention of experts and amateurs.

In short, in each population of *P. lilfordi* we observe an evolutionarily independent history linked to different ecological conditions, extremely variable population sizes and availability of trophic resources, or presence of particular competitors and predators. This situation has led to adaptive responses, in many cases unique to each population, that are resolved in morphological, ethological and ecological features so far only observed in that populations. It is impossible to foresee what the future knowledge of numerous populations holds for which there is no reliable data on ecology and behaviour of their lizards.

Consequently, the recognition of all the populations as ESU implies that each and every one of the populations of the Balearic lizard is fully relevant for its conservation. We do not want to protect only the product of evolution, that is, the extraordinary variability of the polytypic Balearic lizard today observed. We want to preserve the future process of evolution of the Balearic lizard (Moritz, 2002).

The Balearic lizard is listed by IUCN as Endangered (criteria: B1ab (ii) + 2ab (iii), Pérez-Mellado & Martínez-Solano, 2010). Paradoxically, the regional conservation managers of the Balearic Islands consider that the species is only Vulnerable (Viada, 2006). Probably, this unrealistic assessment derives from the fact that some specific populations, such as those of the Dragonera or Cabrera Islands, have a large number of lizards. The complete preservation of all the extant populations will be the only guarantee of conservation of the unique evolutionary process of this species (Pérez-Mellado, 2008).

Table S1. Morphometric and scalation characters of adult males from Menorca coastal islets. For each population and character, the average ± standard error and sample size, is given (see Material and Methods for names of variables).

Population	SVL	Tail	Weight	PL	HH	HW	HLL	LAM	FEM	GUL	DOR	VENT	COLL
Addaia gran	62.07 ± 0.47 (57)	99.4 ± 1.93 (21)	6.01±0.12 (54)	15.51± 0.15 (24)	8.21 ± 0.16 (24)	7.21 ± 0.07 (24)	31.14 ± 0.42 (20)	28.35 ± 0.33 (20)	20.26 ± 0.39 (23)	34.36 ± 0.9 (14)	79.57 ± 1.65 (14)	22.29 ± 0.29 (14)	11.79 ± 0.35 (14)
Addaia petita	61.2 ± 0.77 (40)	102.11± 4.45 (9)	5.96±0.19 (32)	15.46 ± 0.13 (20)	7.39± 0.17 (20)	7 ± 0.08 (20)	31.3 ± 0.6 (8)	29.88 ± 0.44 (8)	21.9 ± 0.6 (10)	35.5 ± 1.23 (10)	78.3 ± 0.83 (10)	21.9 ± 0.41 (10)	11.7 ± 0.26 (10)
Aire	70.34 ± 0.11 (1534)	113.21± 0.53 (628)	10.21±0.05 (1497)	18 ± 0.04 (798)	8.93 ± 0.03 (797)	8.26 ± 0.02 (811)	38.25 ± 0.11 (288)	29.01 ± 0.12 (217)	21.02 ± 0.09 (323)	34.14 ± 0.65 (14)	80.79 ± 0.71 (14)	22.93 ± 0.40 (14)	12.15 ± 0.34 (13)
Codrell Gran	66.07 ± 0.3 (122)	109.74± 1.28 (35)	7.98±0.12 (112)	16.93 ± 0.1 (76)	8.93 ± 0.1 (76)	7.72 ± 0.06 (76)	35.47 ± 0.33 (49)	29.43 ± 0.22 (47)	22.67 ± 0.2 (72)	31.17 ± 0.73 (12)	78.69 ± 1.60 (13)	22.83 ± 0.21 (12)	10.75 ± 0.30 (12)
Codrell Petit	68.58 ± 0.66 (40)	110.92± 2.71 (6)	9.52±0.38 (31)	17.29 ± 0.17 (31)	9.74± 0.13 (31)	8.06 ± 0.11 (31)	36.46 ± 0.7 (17)	28.47 ± 0.39 (17)	22.29± 0.24 (31)	32.50 ± 0.29 (4)	85.25 ± 3.30 (4)	23.75± 0.75 (4)	10.50 ± 0.65 (4)
Bledas	63.88 ± 1.14 (14)	95± 12.5 (2)	6.84±0.51 (13)	15.31 ± 0.58 (7)	8.16± 0.39 (7)	7.17 ± 0.35 (7)	31.75 ± 1.1 (6)	28.14 ± 0.34 (7)	20.12± 0.64 (8)	34.86 ± 1.97 (7)	77.33± 0.42 (6)	22.57 ± 0.65 (7)	11.33 ± 0.56 (6)
Colom	66.78 ± 0.22 (322)	107.5 ± 0.93 (123)	7.93±0.08 (291)	16.96 ± 0.07 (213)	8.67 ± 0.05 (213)	7.64 ± 0.05 (213)	36.33 ± 0.17 (174)	29.26 ± 0.15 (140)	21.93 ± 0.12 (219)	33.62 ± 0.95 (21)	84.20 ± 1.78 (20)	25.05 ± 0.58 (21)	11.38 ± 0.28 (21)
Carbó	62.63 ± 0.49 (30)	103.78± 2.17 (9)	6.49±0.24 (23)	15.52 ± 0.2 (13)	7.35 ± 0.26 (13)	7.29 ± 0.13 (13)	32.02 ± 0.31 (5)	30.8 ± 0.66 (5)	21.6 ± 0.51 (5)	32.60 ± 1.12 (5)	81.00 ± 1.67 (5)	23.80 ± 0.58 (5)	12.60 ± 0.40 (5)
Mel	71.43 ± 0.88 (14)	124.5± 1.44 (3)	9.76±0.41 (14)	17.13 ± 0.36 (11)	7.03 ± 0.22 (11)	7.65 ± 0.17 (11)	38.34 ± 0.56 (2)	28.67 ± 1.2 (3)	20.33 ± 0.33 (3)	35.00 ± 1.00 (3)	92.00 ± 3.79 (3)	26.00 ± 0.58 (3)	11.00 ± 0.00 (3)
Porros	69.32 ± 0.73 (66)	105.48± 4.66 (12)	8.43±0.21 (56)	17.02 ± 0.41 (14)	8.15 ± 0.29 (15)	8.06 ± 0.16 (14)	35.77 ± 1.00 (10)	29 (1)	22 ± 1.00 (2)	27.33 ± 1.86 (3)	70.00 ± 6.08 (3)	22.67 ± 1.20 (3)	11.50 ± 1.50 (2)
Rei	68.31 ± 0.38 (141)	111.95± 1.63 (59)	8.93 ± 0.2 (94)	17.45 ± 0.12 (63)	8.38 ± 0.11 (64)	7.91 ± 0.08 (64)	36.15 ± 0.3 (53)	30.33 ± 0.41 (12)	20.83 ± 0.65 (12)	36.50±1.00 (12)	80.10 ± 1.40 (10)	22.50 ± 0.42 (12)	11.09 ± 0.37 (11)
Rovells	65.24 ± 0.66 (26)	97.38± 6.57 (9)	7.33 ±0.27 (22)	16.13 ± 0.25 (17)	7.49 ± 0.28 (17)	7.52 ± 0.16 (17)	32.89 ± 0.92 (4)	29.75 ± 1.65 (4)	21.6 ± 0.81 (5)	32.80 ± 0.86 (5)	76.20 ± 1.32 (5)	22.40 ± 0.52 (5)	12.00 ± 0.71 (5)
Tusqueta	64.42 ± 0.45 (74)	103.62± 2.32 (12)	7.33 ± 0.1 (58)	16.57 ± 0.08 (60)	8.78 ± 0.09 (60)	7.48 ± 0.05 (60)	34.11 ± 0.31 (52)	27.83 ± 0.19 (47)	21.08 ± 0.21 (59)	30.80 ± 0.66 (10)	81.30 ± 2.31 (10)	21.80 ± 0.42 (10)	10.60 ± 0.31 (10)
Sanitja	63.45 ± 0.35 (70)	93.43± 2.22 (35)	6.65 ±0.16 (51)	16.02 ± 0.12 (36)	8.24 ± 0.11 (36)	7.4 ± 0.1 (36)	32.93 ± 0.31 (33)	28.06 ± 0.39 (33)	21.09 ± 0.28 (35)	33.77 ± 0.52 (26)	76.88 ± 0.90 (26)	22.04 ± 0.18 (26)	11.96 ± 0.25 (25)
Sargantana	62.24 ± 0.61 (61)	95.41± 3.11 (22)	6.71 ± 0.25 (47)	15.31 ± 0.29 (25)	7.54 ± 0.17 (26)	7.09 ± 0.14 (26)	30.42 ± 0.6 (15)	28.27 ± 0.38 (15)	20.47 ± 0.36 (15)	33.93 ± 0.67 (15)	77.21 ± 1.45 (14)	22.40 ± 0.84 (15)	11.36 ± 0.20 (14)
Aguiles	67.81 ± 0.96 (13)	115.5± 4.16 (3)	8.58 ±0.24 (13)	17.01 ± 0.16 (9)	7.58 ± 0.14 (9)	7.77 ± 0.13 (9)	33.73 ± 0.35 (9)	28.36 ± 0.47 (11)	20.55 ± 0.37 (11)	34.45 ± 0.89 (11)	79.82 ± 2.33 (11)	23.55 ± 0.43 (11)	11.80 ± 0.47 (10)
Mones	64.88 ± 0.31 (4)	117.75± 8.25 (2)	7.0 ± 0.51 (4)	15.96 ± 0.19 (4)	7.32 ± 0.13 (4)	7.58 ± 0.22 (4)	34.42 ± 0.73 (4)	32.75 ± 0.95 (4)	22.00 ± 1.78 (4)	40.50 ± 1.19 (4)	91.50 ± 2.33 (4)	23.75 ± 0.48 (4)	10.50 ± 0.50 (2)

Table S2. Morphometric and scalation characters of adult females from Menorca coastal islets. For each population and character, the average ± standard error and sample size, is given (see Material and Methods for names of variables).

Population	SVL	Tail	Weight	PL	HH	HW	HLL	LAM	FEM	GUL	DOR	VENT	COLL
Addaia gran	54.55 ± 0.82 (17)	84.45 ± 3.72 (10)	3.65±0.20 (13)	12.61± 0.14 (5)	6.45±0.11 (5)	5.99 ± 0.13 (5)	26.06 ± 0.58 (5)	28.40 ± 0.75 (5)	19.00 ± 0.71 (5)	32.40 ± 1.29 (5)	73.20 ± 1.88 (5)	24.20 ± 0.86 (5)	12 ± 1.05 (5)
Addaia petita	53.64 ± 0.60 (8)	67.05± 3.19 (5)	3.20±0.12 (5)	12.31 ± 0.10 (5)	6.17± 0.10 (5)	5.69 ± 0.08 (5)	25.48 ± 0.27 (5)	28.25 ± 0.63 (4)	20.60 ± 0.51 (5)	32.20 ± 1.24 (5)	71.60 ± 0.93 (5)	23.80 ± 0.20 (5)	11.60 ± 0.51 (5)
Aire	62.67 ± 0.15 (1153)	98.42± 0.59 (514)	6.46±0.05 (1110)	15.36 ± 0.04 (510)	7.41 ± 0.04 (510)	6.99 ± 0.02 (518)	33.99 ± 0.18 (229)	28.77 ± 0.14 (159)	20.50 ± 0.11 (206)	35.00 ± 0.51 (17)	80.38 ± 1.03 (16)	24.41 ± 0.31 (17)	11.47 ± 0.26 (17)
Codrell Gran	60.04 ± 0.44 (64)	96.05± 1.60 (20)	4.89±0.14 (61)	14.38 ± 0.20 (38)	7.37 ± 0.15 (39)	6.54 ± 0.08 (39)	32.11± 0.41 (35)	29.17 ± 0.23 (30)	21.49 ± 0.27 (35)	32.33 ± 0.58 (9)	78.11 ± 1.35 (9)	24.33 ± 0.33 (9)	9.78 ± 0.32 (9)
Codrell Petit	61.66 ± 0.93 (16)	100.67± 2.03 (6)	5.77±0.32 (15)	14.42 ± 0.21 (10)	7.90± 0.17 (10)	6.69 ± 0.12 (10)	32.56 ± 0.86 (8)	27.71 ± 0.52 (7)	20.80± 0.44 (10)	31.00 ± 2.00 (2)	75.50 ± 0.50 (2)	25.00 ± 0.00 (2)	10.00 ± 1.00 (2)
Bledas	58.22 ± 0.86 (11)	86.25± 4.35 (4)	4.31±0.22 (10)	13.05 ± 0.45 (2)	6.85± 0.15 (2)	6.15 ± 0.05 (2)	29.20 ± 1.40 (2)	27.00 ± 0.26 (6)	20.67± 1.58 (6)	34.00 ± 1.90 (6)	84.25± 7.12 (4)	26.17 ± 1.01 (6)	12.00 ± 2.00 (2)
Colom	60.48 ± 0.26 (238)	92.84 ± 1.04 (107)	5.56 ±0.07 (225)	14.40 ± 0.07 (137)	7.27 ± 0.08 (139)	6.44 ± 0.04 (139)	32.00 ± 0.20 (129)	28.68 ± 0.20 (102)	21.46 ± 0.14 (144)	33.54 ± 0.62 (13)	75.62 ±1.61 (13)	26.92 ± 0.94 (13)	11.15 ± 0.54 (13)
Carbó	57.76 ± 0.50 (18)	91.90± 2.41 (5)	3.64±0.19 (9)	13.18 ± 0.05 (5)	7.19 ± 0.02 (5)	6.53 ± 0.08 (5)	27.01 ± 0.26 (5)	29.25 ± 0.48 (4)	19.75 ± 0.25 (4)	31.20 ± 0.92 (5)	78.60 ± 1.78 (5)	25.00 ±0.45 (5)	12.60 ± 0.68 (5)
Mel	72.00 ± 1.04 (3)	119.50 (1)	8.80 (1)	15.95 ± 0.15 (2)	6.62 ± 0.02 (2)	6.64 ± 0.01 (2)	34.91 ± 0.29 (2)	33.00 ± 1.00 (3)	21.33 ± 0.33 (3)	35.67 ± 0.88 (3)	84.33 ± 2.33 (3)	29.00 ± 0.00 (2)	11.00 ± 0.58 (3)
Porros	63.01 ± 0.53 (42)	94.79± 1.89 (12)	5.93±0.20 (40)	13.85 (1)	6.25 (1)	6.45 (1)	28.21 (1)	29.00 (1)	22 (1)	27 (1)	70 (1)	23 (1)	11 (1)
Rei	62.14 ± 0.43 (102)	99.92± 2.08 (31)	6.03 ± 0.15 (64)	14.83 ± 0.17 (44)	6.91 ± 0.13 (44)	6.71 ± 0.06 (44)	31.69 ± 0.30 (43)	30.67 ± 0.33 (3)	21.67± 0.88 (3)	36.00 ± 2.52 (3)	75.67 ± 1.45 (3)	24.33 ± 0.33 (3)	10.67 ± 0.67 (3)
Rovells	59.78 ± 0.67 (18)	90.62± 6.81 (4)	4.96 ±0.17 (14)	13.85 ± 0.15 (2)	6.97 ± 0.27 (2)	6.67 ± 0.17 (2)	27.93 ± 1.88 (2)	26.50 ± 0.50 (2)	19.00 ± 0.00 (2)	30.50 ± 0.50 (2)	70.50 ± 0.50 (2)	22.50± 0.50 (2)	10.50 ± 0.50 (2)
Tusqueta	57.92 ± 0.59 (20)	90.14± 1.99 (7)	4.74 ±0.18 (20)	13.77 ± 0.10 (18)	7.55 ± 0.12 (18)	6.20 ± 0.08 (18)	30.19 ± 0.39 (15)	27.07 ± 0.40 (14)	20.72 ± 0.39 (18)	29 (1)	62 (1)	25 (1)	12 (1)
Sanitja	56.80 ± 0.67 (12)	81.36± 3.37 (7)	4.33 ±0.29 (7)	12.98 ± 0.25 (3)	6.45± 0.09 (3)	5.98 ± 0.12 (3)	27.38 ± 0.68 (3)	28.00 ± 0.58 (3)	21.33 ± 0.88 (3)	32.00 ± 1.53 (3)	74.33 ± 3.38 (3)	24.00 ± 0.58 (3)	10.33± 0.88 (3)
Sargantana	54.91 ± 0.62 (23)	79.04± 2.38 (12)	3.99 ±0.19 (17)	13.03 ± 0.18 (13)	6.61 ± 0.13 (13)	6.03 ± 0.07 (13)	27.31 ± 0.27 (13)	27.92 ± 0.37 (13)	19.62 ± 0.51 (13)	32.23 ± 0.68 (13)	76.00 ± 1.06 (13)	22.85± 0.44 (13)	10.92 ± 0.31 (13)
Aguiles	60.90 ± 0.99 (5)	94 (1)	5.12 ±0.24 (4)	13.44 ± 0.21 (5)	6.22 ± 0.65 (5)	6.20 ± 0.09 (5)	28.51 ± 1.51 (4)	28.20 ± 0.80 (5)	20.20 ± 0.37 (5)	32.60 ± 1.21 (5)	80.00 ± 3.00 (3)	25.20 ± 0.58 (5)	10.80 ± 0.49 (5)
Mones	58.17 ± 0.73 (3)	117.75 (1)	4.12 ± 0.12 (2)	13.36 (1)	5.89 ± 0.02 (2)	6.20 (1)	29.52 ± 0.05 (2)	33.50 ± 3.50 (2)	22.50 ± 0.50 (2)	34.00 ± 1.00 (2)	93.00 ± 9.00 (2)	25.50 ± 0.50 (2)	9.00 ± 1.00 (2)

Table S3. Morphometric and scalation characters of adult males from Mallorca coastal islands and islets. For each population and character, the average ± standard error and sample size, is given (see Material and Methods for names of variables).

Population	SVL	Tail	Weight	PL	HH	HW	HLL	LAM	FEM	GUL	DOR	VENT	COLL
1. Caragol	67.13 ± 0.76 (25)	109.91 ± 3.97 (4)	8.25 ± 0.19 (19)	16.61± 0.13 (20)	8.12 ± 0.1 (20)	7.68 ± 0.09 (20)	35.43 ± 0.53 (20)	30.63 ± 0.42 (19)	20.9 ± 0.26 (20)	33.94 ± 0.95 (18)	78.25 ± 1.45 (20)	25.61 ± 0.49 (18)	10.85 ± 0.22 (20)
2. Dragonera	70.62 ± 0.24 (205)	128.99 ± 1.79 (43)	10.67 ± 0.13 (197)	17.67 ± 0.09 (87)	8.52± 0.09 (87)	8.23 ± 0.09 (87)	36.93 ± 0.34 (51)	29.2 ± 0.26 (46)	23.25 ± 0.2 (75)	34.86 ± 1.08 (7)	79 ± 1.77 (7)	23.29 ± 0.52 (7)	13.0 ± 0.31 (7)
3. Colomer	74.42 ± 0. 58 (42)	132 ± 2.06 (7)	12.05 ± 0.29 (17)	18.03 ± 0.15 (40)	8.79 ± 0.12 (41)	8.3 ± 0.08 (40)	39.5 ± 0.29 (36)	30.88 ± 0.42 (34)	22.92 ± 0.4 (38)	35.72 ± 0.77 (25)	93.23 ± 1.62 (26)	23.96 ± 0.23 (26)	11.68 ± 0.23 (25)
4. Malgrat gran	70.83 ± 0.57 (12)	124.24 ± 4.52 (7)	10.26 ± 0.56 (10)	17.28 ± 0.36 (10)	7.81 ± 0.25 (10)	8.18 ± 0.18 (10)	37.31 ± 0.63 (10)	28 ± 0.38 (11)	20.73 ± 0.33 (11)	30.82 ± 0.57 (11)	74.6 ± 2.49 (10)	22.1 ± 0.59 (10)	12.27 ± 0.27 (11)
5. Malgrat petit	76.0 ± 2.57 (3)	122 (1)	12 ± 1.53 (3)	14.45 (1)	5.95 (1)	6.74 (1)	32.71 (1)	28 (1)	23 (1)	32 (1)	83 (1)	32 (1)	11 (1)
6. Moltona	70.48 ± 0.7 (21)	135 (1)	9.8 ± 0.42 (21)	17.79 ± 0.17 (14)	8.37± 0.17 (14)	8.3 ± 0.12 (14)	35.23 ± 0.33 (6)	32.33 ± 0.21 (6)	22 ± 0.63 (6)	36 ± 1.26 (6)	82.67± 0.56 (6)	23.33 ± 0.21 (6)	11.67 ± 0.56 (6)
7. Guardia	63.38 ± 0.57 (40)	112.34 ± 3.59 (16)	7.35 ± 0.2 (34)	15.89 ± 0.17 (26)	7.32 ± 0.13 (26)	7.51 ± 0.14 (26)	32.08 ± 0.71 (19)	30.2 ± 0.39 (15)	20 ± 0.48 (15)	34.47 ± 1.09 (15)	79.93 ± 1.49 (14)	23.13 ± 0.46 (15)	11.2 ± 0.33 (15)
8. Pelada	65.8 ± 0.61 (27)	119.06 ± 6.71 (8)	8 ± 0.22 (27)	16.5 ± 0.17 (22)	7.31 ± 0.1 (22)	7.29 ± 0.08 (22)	36.45 ± 0.4 (12)	28.17 ± 0.41 (12)	16.83 ± 0.32 (12)	35.83 ± 1.05 (12)	82.17 ± 1.38 (12)	23.5 ± 0.38 (12)	10 ± 0.37 (6)
9. Toro	69.19 ± 0.52 (8)	121 ± 3.06 (3)	9.86 ± 0.3 (7)	16.57 ± 0.2 (7)	7.74 ± 0.16 (7)	7.89 ± 0.23 (7)	34.67 ± 0.53 (6)	27.25 ± 0.48 (4)	20.14 ± 0.7 (7)	32.5 ± 0.56 (6)	80.4 ± 3.23 (5)	23.33 ± 0.21 (6)	13.17 ± 0.31 (6)

Table S4. Morphometric and scalation characters of adult females from Mallorca coastal islands and islets. For each population and character, the average ± standard error and sample size, is given (see Material and Methods for names of variables).

Population	SVL	Tail	Weight	PL	HH	HW	HLL	LAM	FEM	GUL	DOR	VENT	COLL
1. Caragol	61.78 ± 0.74 (20)	99.16 ± 0.89 (8)	5.63 ± 0.17 (14)	14.13± 0.24 (15)	6.60 ± 0.15 (15)	6.57 ± 0.10 (15)	29.84 ± 0.56 (15)	29.87 ± 0.88 (15)	20.47 ± 0.53 (15)	30.13 ± 0.71 (15)	78.47 ± 2.54 (15)	26.73 ± 0.34 (15)	10.60 ± 0.25 (15)
2. Dragonera	62.78 ± 0.30 (124)	111.86 ± 1.63 (40)	6.43 ± 0.11 (117)	14.95 ± 0.13 (46)	7.05± 0.21 (46)	6.74 ± 0.10 (46)	31.91 ± 0.43 (40)	28.32 ± 0.31 (38)	22.62 ± 0.37 (45)	35.00 ± 4.00 (2)	64 (1)	22.50 ± 0.50 (2)	10.50 ± 0.50 (2)
3. Colomer	67.58 ± 1.09 (19)	113.17±11.37 (3)	7.67 ± 0.55 (13)	15.50 ± 0.19 (12)	6.98 ± 0.17 (12)	6.93 ± 0.09 (12)	34.48 ± 0.60 (11)	30.67 ± 0.40 (12)	22.36 ± 0.75 (11)	32.83 ± 1.25 (6)	84.83 ± 1.40 (6)	26.00 ± 0.26 (6)	10.67 ± 0.61 (6)
4. Malgrats gran	64.79 ± 1.07 (12)	113.43 ± 3.45 (7)	6.92 ± 0.29 (12)	14.22 ± 0.32 (5)	6.42 ± 0.15 (5)	6.91 ± 0.24 (5)	31.56 ± 0.60 (5)	28 ± 0.71 (5)	19.67 ± 0.84 (6)	30.17 ± 0.91 (6)	74.00 ± 1.29 (6)	24.00 ± 1.06 (6)	11.83 ± 0.31 (6)
5. Malgrat petit	64.78 ± 2.66 (9)	108.0 ± 3.77 (3)	6.22 ± 0.34 (8)	14.45 (1)	5.95 (1)	6.74 (1)	32.71 (1)	28.00 (1)	23 (1)	39 (1)	83 (1)	30 (1)	11 (1)
6. Moltona	59.70 ± 1.02 (15)	108.50 ± 3.50 (2)	5.03 ± 0.16 (17)	13.52 ± 0.31 (10)	5.45 ± 0.11 (10)	6.20 ± 0.16 (10)	28.29 ± 0.48 (10)	31.43 ± 0.57 (14)	20.14 ± 0.52 (14)	35.71 ± 1.03 (14)	87.17± 0.82 (12)	26.14 ± 0.35 (14)	10.43 ± 0.25 (14)
7.Guardia	58.60 ± 0.65 (20)	98.67 ± 6.33 (3)	5.35 ± 0.27 (17)	14.20 ± 0.32 (11)	6.17 ± 0.19 (11)	6.47 ± 0.17 (9)	29.46 ± 0.84 (11)	31.64 ± 0.62 (11)	19.73 ± 0.59 (11)	35.10 ± 1.39 (10)	77.00 ± 1.62 (11)	25.36 ± 0.41 (11)	12.11 ± 0.31 (9)
8. Pelada	62.12 ± 1.04 (13)	107.12 ± 6.78 (4)	6.26 ± 0.25 (11)	14.07 ± 0.30 (8)	6.32 ± 0.19 (8)	6.28 ± 0.19 (8)	29.82 ± 0.32 (6)	30.00 ±0.0 (6)	16.00 ± 0.38 (8)	35.50 ± 1.52 (8)	81.25 ± 2.09 (8)	25.75 ± 0.16 (8)	10.50 ± 0.29 (4)
9. Toro	62.09 ± 0.93 (11)	110.75 ± 2.25 (2)	5.79 ± 0.16 (11)	13.65 ± 0.14 (8)	6.10 ± 0.12 (8)	6.81 ± 0.08 (8)	31.65 ± 0.68 (8)	27.40 ± 0.51 (5)	20.62 ± 0.96 (8)	32.00 ± 0.68 (6)	74.83 ± 1.14 (6)	25.50 ± 0.34 (6)	12.00 ± 0.37 (6)

Table S5. Morphometric and scalation characters of adult males from Cabrera archipelago. For each population and character, the average ± standard error and sample size, is given (see Material and Methods for names of variables).

Popuation	SVL	Tail	Weight	PL	HH	PW	HLL	LAM	FEM	GUL	DOR	VEN	COLL
Cabrera	66.6 ± 0.51 (75)	112.47 ± 4.23 (17)	8.91 ± 0.3 (34)	16.87± 0.16 (30)	7.77 ± 0.13 (30)	7.99 ± 0.2 (30)	35.06 ± 0.44 (28)	30.64 ± 0.52 (14)	22.6 ± 0.39 (15)	33.07 ± 1.09 (15)	82.22 ± 3.18 (9)	23.13 ± 0.41 (15)	11.27 ± 0.4 (15)
Conillera	68.79 ± 0.84 (21)	115.1 ± 4.82 (5)	10.25 ± 0.26 (4)	16.8 ± 0.25 (17)	8.76 ± 0.2 (17)	7.75 ± 0.18 (17)	35.85 ± 0.58 (17)	32.41 ± 0.44 (17)	23.47 ± 0.45 (17)	32.53 ± 0.65 (17)	80.0 ± 1.6 (17)	22.29 ± 0.27 (17)	11.18 ± 0.4 (17)
Esponja	71.08 ± 1.32 (6)	132 (1)	8.6 ± 0.37 (5)	17.29 ±0.22 (4)	7.37 ± 0.16 (4)	7.78 ± 0.15 (4)	37.74 ± 1.71 (4)	29 ± 1.05 (5)	21.2 ± 0.8 (5)	28.2 ± 1.91 (5)	86.75 ± 1.11 (4)	24.4 ± 1.03 (5)	10.4 ± 0.51 (5)
Estell des Coll	77.18 ± 0.89 (19)	135.88± 3.54 (4)	12.8 ± 0.73 (5)	18.03 ±0.16 (14)	9.52 ± 0.15 (14)	8.39 ± 0.16 (14)	40.53 ± 0.33 (14)	32.5 ± 0.36 (14)	22.64 ± 0.57 (14)	31.86 ± 0.94 (14)	80.07 ± 0.62 (14)	21.71 ± 0.37 (14)	11.57 ± 0.17 (14)
Estell de Fora	78.67 ± 2.03 (3)	139.67 ± 4.67 (3)	14 ± 0.00 (2)	19.08 (1)	10.97 (1)	9.56 (1)	41.7 (1)	31 (1)	21 (1)	29 (1)	77 (1)	23 (1)	13 (1)
Fonoll Gros	68.92 ± 0.91 (20)	117.5 ± 5.86 (8)	10.34 ± 0.91 (9)	17.09 ±0.23 (11)	8.77 ± 0.16 (11)	7.76 ± 0.09 (14)	35.74 ± 0.57 (10)	30.8 ± 0.49 (10)	22.64± 0.6 (14)	30.64 ± 0.83 (11)	74.55± 0.82 (11)	22 ± 0.23 (11)	11.45 ± 0.31 (11)
Fonoll Petit	67.67 ± 0.88 (3)	115 (1)	11 (1)	16.88 ±0.29 (3)	8.57 ± 0.23 (3)	7.6 ± 0.09 (3)	36.01 ± 1.29 (3)	29 ± 2.52 (3)	21.67 ± 0.88 (3)	31.33 ± 0.33 (3)	73.33 ± 1.2 (3)	22 ± 0.58 (3)	11.33 ± 0.67 (3)
Imperial	75.76 ± 0.63 (25)	132.3 ± 4.08 (5)	10.56 ± 1.17 (8)	18.19 ±0.12 (19)	9.57 ± 0.12 (19)	8.72 ± 0.12 (19)	39.32 ± 0.48 (18)	30.06 ± 0.34 (18)	22.05 ± 0.44 (19)	31.79 ± 0.66 (19)	77.26 ± 1.17 (19)	22.32 ± 0.23 (19)	11.79 ± 0.36 (19)
Foradada	70.38 ± 0.45 (85)	112.85 ± 2.66 (39)	10.47 ± 0.41 (33)	17.63 ± 0.2 (48)	8.59 ± 0.1 (48)	8.42 ± 0.1 (55)	37.35 ± 0.32 (46)	30.68 ± 0.5 (31)	20.73 ± 0.2 (41)	28.03 ± 0.61 (33)	75.67 ± 1.78 (27)	22.12 ± 0.23 (33)	11.55 ± 0.19 (33)
Plana	67.22 ± 0.73 (20)	115.6 ± 3.52 (5)	11.1 ± 0.22 (5)	16.36 ±0.16 (15)	8.15 ± 0.16 (15)	7.42 ± 0.14 (15)	33.84 ± 0.48 (15)	29.53 ± 0.41 (15)	20.4 ± 0.43 (15)	29.8 ± 0.49 (15)	71.33 ± 0.68 (15)	20.87 ± 0.19 (15)	11.13 ± 0.24 (15)
Pobra	68.44 ± 0.52 (43)	121.27 ± 2.84 (11)	11.5 ± 1.0 (5)	16.92 ±0.11 (38)	8.97 ± 0.09 (38)	8.12 ± 0.08 (38)	35.59 ± 0.37 (37)	29.62 ± 0.31 (37)	20.5 ± 0.31 (38)	29.16 ± 0.33 (38)	74.03 ± 0.57 (34)	21.61 ± 0.16 (38)	11.92 ± 0.19 (38)
Redona	71.08 ± 0.75 (26)	121 ± 4.43 (6)	9.8 ± 0.56 (10)	17.15 ±0.19 (16)	8.53 ± 0.16 (16)	7.81 ± 0.16 (16)	36.6 ± 0.42 (16)	31.19 ± 0.44 (16)	21.5 ± 0.46 (16)	29.56 ± 0.56 (16)	76.94 ± 0.84 (16)	21.62 ± 0.3 (16)	11.38 ± 0.27 (16)
Ses Bledes	74.63 ± 1.02 (15)	127.83 ± 3.88 (6)	14.07 ± 0.63 (12)	18.44 ±0.28 (7)	8.65 ± 0.35 (7)	8.72 ± 0.16 (7)	41.31 ± 0.98 (7)	31.71 ± 0.68 (7)	20.57 ± 0.65 (7)	31.86 ± 0.55 (7)	78.67 ± 1.2 (3)	22.29 ± 0.75 (7)	10.57 ± 0.3 (7)
Rates	69.58 ± 1.24 (13)	118 (1)	9.9 ± 0.64 (10)	16.11 ±0.17 (3)	8.1 ± 0.16 (3)	6.84 ± 0.2 (3)	34.7 ± 0.33 (3)	31.67 ± 0.33 (3)	22 ± 0.55 (5)	32.25 ± 3.07 (4)	74.67 ± 2.6 (3)	22.67 ± 0.33 (3)	11 ± 1.0 (3)
Esclatasang	72.83 ± 0.44 (39)	129.72 ± 3.11 (9)	11.49 ± 0.28 (27)	17.87 ±0.19 (23)	8.75 ± 0.27 (23)	8.35 ± 0.12 (26)	37.77 ± 0.48 (22)	30.11 ± 0.54 (9)	21.41 ± 0.45 (17)	32 ± 0.36 (18)	75.4 ± 1.17 (10)	23.22 ± 0.38 (18)	11.88 ± 0.26 (17)
Xapat Gros	73.26 ± 0.48 (23)	131.83 ± 3.94 (6)	13.64 ± 0.8 (7)	18.1 ± 0.12 (21)	9.75 ± 0.11 (21)	8.79 ± 0.1 (21)	39.44 ± 0.57 (19)	30.39 ± 0.33 (18)	20.9 ± 0.4 (21)	30.38 ± 0.49 (21)	75.95 ± 0.72 (20)	22.05 ± 0.2 (21)	11.38 ± 0.29 (21)
Xapat Petit	70.62 ± 1.61 (8)	132 (1)	7.83 ± 0.96 (3)	18.13 ±0.28 (8)	9.47 ± 0.26 (8)	8.51 ± 0.16 (8)	38.7 ± 0.6 (7)	30.57 ± 0.57 (7)	20.38 ± 0.5 (8)	31.38 ± 0.68 (8)	73.38 ± 1.19 (8)	22.12 ± 0.4 (8)	11.75 ± 0.45 (8)

Table S6. Morphometric and scalation characters of adult males from Cabrera archipelago. For each population and character, the average ± standard error and sample size, is given (see Material and Methods for names of variables).

Population	SVL	Tail	Weight	PL	HH	HW	HLL	LAM	FEM	GUL	DOR	VENT	COLL
Cabrera	59.98 ± 0.44 (84)	104.59 ± 2.18 (23)	5.91 ± 0.27 (23)	14.22±0.12 (48)	6.83 ± 0.08 (50)	6.67 ±0.1 (49)	30.62 ± 0.26 (50)	31.70 ± 0.27 (43)	21.47 ± 0.34 (43)	30.07 ± 0.36 (42)	74.80 ± 0.62 (40)	24.40 ± 0.21 (43)	11.49 ± 0.19 (43)
Conillera	61.66 ± 0.70 (16)	85 (1)	6.65 ± 0.15 (2)	14.36 ± 0.19 (14)	7.23 ± 0.18 (14)	6.4±0.07 (14)	31.35 ± 0.45 (14)	30.14 ± 0.56 (14)	22.36 ± 0.55 (14)	32.14 ± 0.53 (14)	77.93 ± 1.02 (14)	24.21 ± 0.26 (14)	11.14 ± 0.36 (14)
Esponja	65.90 ± 1.21 (5)	87 ± 8.00 (2)	5.75 ± 0.43 (4)	14.56 ± 0.31 (2)	6.49 ± 0.51 (2)	6.64±0.34 (2)	32.22 ± 2.07 (2)	29.33 ± 0.33 (3)	19.00 ± 0.58 (3)	28.33 ± 1.2 (3)	82.33 ± 4.84 (3)	25.0 ± 1.53 (3)	11.0 ± 0.00 (3)
Estell des Coll	70.82 ± 2.10 (9)	112.5± 4.5 (2)	8.38 ± 0.75 (4)	15.22 ± 0.32 (5)	7.67 ± 0.29 (5)	7.38 ± 0.28 (5)	35.62 ± 0.60 (5)	32.2 ± 0.86 (5)	22.40 ± 0.81 (5)	28.40 ± 0.81 (5)	76.20 ± 2.11 (5)	24.40 ± 0.40 (5)	11.00 ± 0.45 (5)
Estell de Fora	65.36 ± 1.36 (7)	121.88 ± 1.71 (4)	5 (1)	15.83 ± 0.22 (6)	7.85 ± 0.19 (6)	7.38 ± 0.10 (6)	37.62 ± 0.44 (6)	31.0 ± 0.45 (6)	20.17 ± 0.48 (6)	31.00 ± 0.26 (6)	79.00 ± 2.28 (6)	25.17 ± 0.65 (6)	11.67 ± 0.56 (6)
Fonoll Gros	60.43 ± 0.93 (15)	112.75 ± 12.75 (2)	8.33 ± 1.10 (3)	14.09 ± 0.14 (12)	6.66 ± 0.11 (12)	6.45 ± 0.21 (13)	30.73 ± 0.34 (12)	31.67 ± 0.56 (12)	21.92 ± 0.57 (12)	31.58 ± 0.60 (12)	74.50± 0.96 (12)	23.17 ± 0.34 (12)	11.17 ± 0.34 (12)
Fonoll Petit	61.10 ± 2.14 (5)	115 (1)	11 (1)	14.67 ± 0.34 (5)	6.92 ± 0.12 (5)	6.70 ± 0.17 (5)	31.89 ± 0.43 (5)	30.80 ± 0.37 (5)	22 ± 0.71 (5)	30.20 ± 1.07 (5)	75.60 ± 1.17 (5)	24.00 ± 0.55 (5)	11.00 ± 0.71 (5)
Imperial	67.71 ± 1.29 (14)	114.60 ± 6.9 (5)	5.50 ± 1.5 (2)	15.64 ± 0.29 (13)	7.90 ± 0.18 (13)	7.38 ± 0.15 (13)	35.48 ± 0.86 (13)	30.08 ± 0.52 (13)	20.92 ± 0.43 (13)	31.23 ± 0.91 (13)	73.31±1.31 (13)	22.85 ± 0.37 (13)	11.00 ± 0.52 (13)
Foradada	65.86 ± 0.69 (33)	107.15 ± 2.54 (10)	6.79 ± 0.30 (14)	15.70 ± 0.24 (23)	7.21 ± 0.12 (23)	7.23 ± 0.13 (24)	32.68±0.58 (21)	29.12 ± 0.40 (17)	19.50 ± 0.37 (18)	28.78 ± 0.64 (18)	70.50 ± 1.59 (14)	23.56 ± 0.32 (18)	11.50 ± 0.31 (18)
Plana	59.53 ± 0.66 (19)	96.50 ± 1.26 (3)	6.22 ± 0.56 (4)	14.15 ± 0.25 (15)	6.71 ± 0.11 (15)	6.30 ± 0.14 (15)	29.86 ± 0.58 (13)	28.92 ± 0.67 (13)	20.0 ± 0.38 (15)	29.07 ± 0.69 (15)	71.07 ± 0.99 (15)	22.80 ± 0.34 (15)	11.07 ± 0.33 (15)
Pobra	61.70 ± 0.52 (30)	86.75 ± 14.48 (4)	8.70 ± 0.68 (4)	14.43 ± 0.15 (25)	7.41 ± 0.10 (25)	6.87 ± 0.07 (25)	31.44 ± 0.32 (25)	29.12 ± 0.35 (25)	20.12 ± 0.40 (25)	29.36 ± 0.43 (25)	72.42 ± 0.84 (19)	22.92±0.26 (25)	11.40 ± 0.31 (25)
Redona	63.62 ± 1.06 (8)	121 (1)	9.80 (1)	14.48 ± 0.19 (8)	6.72 ± 0.12 (8)	6.30 ± 0.10 (8)	32.15 ± 0.55 (8)	31.38 ± 0.50 (8)	21.50 ± 0.71 (8)	30.25 ± 0.92 (8)	72.88 ± 0.79 (8)	23.88 ± 0.35 (8)	11.00 ± 0.33 (8)
Ses Bledes	69.45 ± 1.24 (10)	113.50 (1)	8.78 ± 0.50 (8)	15.65 ± 0.27 (8)	6.85 ± 0.25 (8)	7.46 ± 0.12 (8)	36.06 ± 0.66 (8)	31.12 ± 0.93 (8)	21.12 ± 0.52 (8)	33.88 ± 0.52 (8)	78.00 ± 4.00 (2)	25.38 ± 0.37 (8)	11.12 ± 0.23 (8)
Rates	62.05 ± 0.93 (10)	111.50 (1)	6.83 ± 1.36 (3)	14.22 ± 0.14 (8)	6.66 ± 0.06 (8)	6.24 ± 0.09 (8)	30.18 ± 0.46 (8)	32.00 ± 0.53 (8)	22.00 ± 0.58 (10)	30.25 ± 0.59 (8)	73.50 ± 0.96 (8)	25.12 ± 0.48 (8)	11.50 ± 0.33 (8)
Esclatasang	66.18 ± 0.85 (28)	106.25 ± 3.38 (4)	8.50 ± 0.40 (16)	15.02 ± 0.25 (14)	7.27 ± 0.15 (14)	7.09 ± 0.12 (17)	32.29 ± 0.67 (14)	28.50 ± 0.60 (10)	19.91 ± 0.55 (11)	31.27 ± 0.91 (11)	72.60 ± 0.86 (10)	24.00 ± 0.56 (11)	12.45 ± 0.51 (11)
Xapat Gros	62.50 ± 1.13 (6)	115.50 (1)	6.40 ± 0.21 (4)	15.17 ± 0.28 (3)	7.45 ± 0.17 (3)	7.25 ± 0.09 (3)	34.61 ±1.74 (3)	29.67 ± 0.33 (3)	21.67 ± 2.03 (3)	29.67 ± 1.20 (3)	75.67 ± 2.03 (3)	22.67 ± 0.88 (3)	12.00 ± 1.00 (3)
Xapat Petit	61.50 ±1.55 (5)	164.10 (1)	7.83 (1)	14.73 ± 0.39 (5)	7.21 ± 0.31 (5)	6.82 ± 0.22 (5)	33.91 ± 1.22 (5)	30.40 ± 1.17 (5)	18.60 ± 0.40 (5)	29.80 ± 0.73 (5)	73.80 ± 1.53 (5)	22.00 ± 0.32 (5)	11.75 ± 0.75 (4)

Table S7. Body dimensions and scalation traits for adult males and females of *P. lilfordi.* Mean ± SE and sample sizes are given. We also give F values, degrees of freedom (d.f.) and p-values for ANOVA analysis of log (SLV) and for minimum significant models of ANCOVA analyses of the remaining log-transformed values of morphological traits under study.

Trait	Males Mean ± SE (n)	Females Mean ± SE (n)	F-value	d.f.	p-value	F-value of interaction	p-value of interaction
SVL	68.95 ± 0.08 (3427)	62.07 ± 0.1 (2295)	2662.0	1, 5270	2×10^{-16}		
Weight	9.44 ± 0.04 (2927)	6.17 ± 0.04 (1931)	8203.49	1, 4843	2.2×10^{-16}	67.321	2.929×10^{-16}
TL	112.36 ± 0.42 (1207)	98.29 ± 0.47 (882)	681.67	1, 2081	2×10^{-16}	6.3169	0.01203
PL	17.43 ± 0.03 (1897)	14.87 ± 0.03 (1126)	6993.66	1, 3016	2.2×10^{-16}	58.815	2.321×10^{-14}
HH	8.68 ± 0.02 (1900)	7.2 ± 0.03 (1132)	2350.84	1, 3025	2.2×10^{-16}	15.814	7.151×10^{-5}
HW	8.02 ± 0.02 (1925)	6.79 ±0.02 (1141)	4292.36	1, 3059	2.2×10^{-16}	47.028	8.433×10^{-12}
HLL	36.59 ± 0.09 (1149)	32.23± 0.1 (817)	1602.85	1, 1959	2.2×10^{-16}	19.387	1.125×10^{-5}
lamellae	29.47 ± 0.07 (936)	29.35 ± 0.09 (663)	1.8352	1, 1592	0.1757	4.209	0.04037
femoralia	21.49 ± 0.06 (1250)	20.9 ± 0.07 (782)	27.43	2, 2024	1.754×10^{-12}	0.0498	0.823
gularia	32.46 ± 0.18 (493)	31.62 ± 0.18 (357)	9.591	1, 843	0.002	8.17	0.004
dorsalia	78.69 ± 0.34 (458)	76.12 ± 0.34 (334)	27.556	1, 784	1.967×10^{-7}	0.292	0.5885
ventralia	22.73 ± 0.08 (492)	24.4 ± 0.1 (358)	173.699	1, 843	2.2×10^{-16}	3.0747	0.0798
collaria	11.55 ± 0.06 (478)	11.2 ± 0.07 (348)	17.12	1, 819	3.872×10^{-5}	0.0017	0.9671

Table S8. GenBank accession numbers of populations included in the genetic study of *Podarcis lilfordi.*

Population/ Gene	12S RNA	RC	CYTB/1	CYTB/2	NADH	COI	MC1R
Colomer	EF694760	EF694775/76	EF990524/25	EF694801/02	EU006737-39	MT044522/3	JX126659-63
Dragonera	EF694761	EF694773	EF990519-21	EF694799	EU006730-33	MT044524/5	JX126624/28-32
Malgrat Petit	EF694762	EF694774	EF990522	EF694799	EU006734	MT044526	JX126637
Malgrat Gran	EF694762	EF694774	EF990522	EF694799	EU006734	MT044526	JX126625/36
Toro	EF694761	EF694774	EF990523	EF694800	EU006735	MT044527	JX126633-35
Caragol	EF694760	EF694771	EF990517	KF003362	EU006728	MT044521	MT044521
Guardia	EF694760	EF694771	EF990517	KF003362	EU006728	MT044521	JX126627/64/65
Moltona	EF694760	EF694771/72	EF990517/18, EF694798	KF003362	EU006728/29	MT044519/21	JX126626/66/67
Pelada	EF694760	EF694771	EF990517	KF003362	EU006728	MT044521	
Colonia de Sant Jordi						MT044510	
Porrassa	EF694760	EF694772	EF990518	EF694798	EU006736	MT044529	
Aire	EF694766	EF694787	EF990546	EF694810	EU006756	MT044528	JX126638/39
Addaia Gran	EF694766	EF694786/87	EF990543/44	EF694809	EU006756	MT044529/30	
Addaia Petita	EF694766	EF694787/88	EF990543/45	EF694809	EU006756	MT044529	JX126647
Colom	EF694766	EF694787/88	EF990543/49	EF694809	EU006756	MT044533	
Bledes Menorca	EF694766	EF694787	EF990543	EF694812	EU006756	MT044529	
Codrell Gran	EF694766	EF694788	EF990543	EF694809	EU006756	MT044531	JX126641/42
Codrell Petit	EF694766	EF694788	EF990543	EF694809	EU006756	MT044531	JX126643-45
Carbó	EF694766	EF694788/89	EF990543/47/48	EF694809/11	EU006756	MT044529/34	
Mel	EF694766	EF694786/88	EF990543	EF694809/13	EU006761/63	MT044529	
Porros	EF694766	EF694788	EF990543	EF694809	EU006758	MT044529	
Rovells	EF694766	EF694787/88	EF990543	EF694809	EU006756/58	MT044529	
Sargantana	EF694766	EF694787/92	EF990543	EF694809	EU006761-63	MT044529	
Tusqueta	EF694766	EF694788	EF990543	EF694809	EU006758/63	MT044532	
Sanitja	EF694766	EF694790/91	EF990543	EF694809	EU006760/61	MT044529	
Aguiles	EF694766	EF694787	EF990543	EF694809	EU006757/63	MT044529	
Mones						MT044529	
Rei	EF694766	EF694787	EF990550/51	EF694810	EU006756/59	MT044529	JX126646
Cabrera Far	EF694764	EF694782	EF990531/36	AM747719	EU006743/45	MT044510/14	JX126623/48-51
Cabrera Port	EF694760/64	EF694772/80/81	EF990531-34	AM747719, EF694806, KF003362	EU006736, EU006743/44	MT044510	JX126652-54
Morro d'en Tià						MT044510	
Miranda	EF694760/64	EF694772/82/83	EF990532/37	KF003362	EU006746-48	MT044510/19	
Foradada	EF694760/63	EF694777	EF990526	EF694803	EU006740	MT044517	JX126655-58
Pobre	EF694760	EF694772	EF990527	KF003362	EU006736	MT044519	
Plana	EF694760	EF694772/78	EF990526	EF694798/804, KF003362	EU006729/36, EU006741	MT044519	
Esponja	EF694760	EF694779	EF990528	EF694804	EU006741	MT044515	
Redona	EF694760	EF694772	EF990526/29	KF003362	EU006742	MT044520	
Conillera	EF694760	EF694772	EF990526	KF003362	EU006736	MT044516/18	
Rates	EF694764	EF694782	EF990538	AM747719	EU006743	MT044513	
Ses Bledes	EF694764	EF694782	EF990531	AM747719	EU006743/49	MT044512	
Fonoll	EF694764	EF694782/84	EF990532	AM747719	EU006743/50	MT044510	
Imperial	EF694764	EF694782	EF990531	AM747719	EU006743/51/52	MT044510	
Estell des Coll	EF694764	EF694782	EF990531	AM747719	EU006743/55	MT044510	
Estell de Fora	EF694765	EF694785	EF990536	EF694807	EU006753	MT044509	

Xapat	EF694765	EF694785	EF990536/39	EF694807	EU006753	MT044509
Esclatasang	EF694764/65	EF694785	EF990541/42	EF694807/08	EU006753/54	MT044509/11
Outgroup						
Na Gorra						MT044506
Espardell	EF694769	EF694794	JX852083	JX852082	JX852121	MT044505
Espalmador	EF694768	EF694794	JX852083	JX852048	JX852121	MT044505
Caragoler	EF694768	EF694794	JX852060	JX852092	JX852124	MT044505
Alga	EF694768	EF694794	JX852083	JX852075	JX852119	MT044505
Negra Nord	EF694768	EF694794	JX852072	JX852092	JX852132	MT044505
Porcs						MT044505
Eivissa	EF694768	EF694794	JX852056	JX852056	JX852121	MT044507
Es Vedrà	EF694768	JX852107				MT044508
Formentera (Sant Francesc Xavier)	EF694768	EF694794	JX852052	JX852082	JX852129	MT044505
Formentera (Punta Trocadors)	EF694768	EF694794	JX852083	JX852082	JX852121	MT044505

CHAPTER 3

Melanism

Preliminary results of MC1R expression in melanic and non-melanic lizards (*Podarcis lilfordi*)

Introduction

This chapter shows the preliminary results of gene expression analysis of the MC1R gene in different development stages in melanic and non-melanic *P. lilfordi* populations. Changes in sequence and studies of expression of the MC1R gene have been related to different pigmentation patterns and to melanism in different species. Most of the gene expression studies were carried out in adult stages, but little is known about the expression of the gene in other stages of development. In Balearic *Podarcis*, a previous study (Buades et al., 2013) found that there was no relationship between MC1R polymorphism and melanic phenotype.

Nineteen egg samples with different incubation times were used from two *P. lilfordi* populations from the island of Menorca, one melanic (Aire Island) and one non-melanic (Colom Island). Moreover, 21 tail samples of adult individuals from melanic and non-melanic populations were used in this analysis. Melanic populations were from Foradada and Esclatasang islands in the Cabrera archipelago, while non-melanic populations were from the main island of Cabrera, near the harbour, and from the island of Dragonera.

Gene expression analysis in all these samples was carried out by means of a RT-qPCR quantification with the aim to determine: a) whether MC1R levels are higher in melanic than in non-melanic populations, as has been described in other species; and b) whether there are differences in the levels of MC1R expression along the embryonic development.

Results

Analysis of expression level of MC1R by RT-qPCR in adult stages

Of the 33 total samples, 21 were extracted successfully with good quality ratios and showed amplification bands in agarose gel. Of these 21, 18 were amplified in RT-qPCR (one from Foradada Island, three from Esclatasang Island, seven from Cabrera harbour, and seven samples from Dragonera Island). A standard curve based on the non-melanic form from Rei (Menorca) for target (MC1R) and reference gene (ACTB) is shown in Figure 1. Efficiency values were 1.774 and 1.833, respectively.

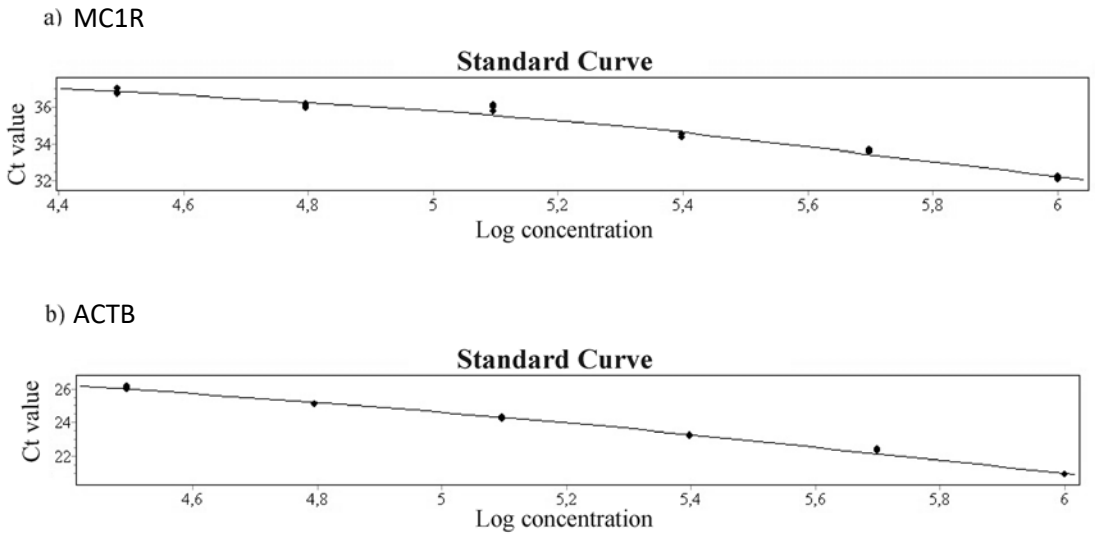


Figure 1. Standard curve for target (MC1R) and reference (ACTB) genes based on non-melanic sample from the Rei (Menorca) population for the analysis based on tail samples.

The fractional cycle numbers of the threshold value (Ct) obtained from the RT-qPCR are indicated in Table 1. One of the three Ct replicate values was eliminated (indicated in red in Table 1) so as to obtain a standard deviation (SD) value of less than 0.30 in target and reference gene. The reference gene, ACTB, showed a constitutive gene expression with a range of Ct values from 23.23-27.74 and with no significant differences between melanic and non-melanic samples. The MC1R gene expressed a lower number of mRNA molecules than the ACTB gene. MC1R Ct values ranged from 36.1-37.86 in melanic individuals and from 34.53-39.1 in non-melanic samples (Table 1).

RT-qPCR analysis of MC1R expression ($2^{-(\Delta\Delta CT)}$) from non-melanic and melanic lizard populations based on adult stages is shown in Figure 2. Fold changes ranged from 0.62 to 8.59 in melanic samples, and a narrower interval was found in non-melanic populations (0.12-1.86), excluding the two outliers (4.32 and 6.67). The mean for melanic samples was higher (4.44) than the average of non-melanic ones (1.45). Similar results were found using the Livak & Schmittgen (2001) and Pfaff (2001) methods.

Table 1. Ct values, mean Ct, standard deviation (SD) for reference gene (ACTB) and target gene (MC1R). Differences between target Ct and reference Ct (ΔCt) and differences between ΔCt of samples and ΔCt of calibrator sample (D10) are indicated. Livak and Pfaff values are also shown. Red values were eliminated to ensure a standard deviation < 0.30 (Taylor, 2009) between the three replicates. Calibrator samples are highlighted in bold.

Sample	ACTB reference			MC1R target			ΔCt	ΔΔCt		
	Ct	Mean Ct	SD	Ct	Mean Ct	SD	Ct(target)-Ct(ref)	ΔCt-ΔCt(calibrator)	Livak	Pfaff
	23,56	23,40	0,15	35,01	34,85	0,23	11,45	1,57	0,34	0,39
D9r	23,39			34,69						
	23,26									
	23,39	23,27	0,12	32,91	34,53	0,01	11,26	1,39	0,38	0,44
Cp1r	23,27			34,54						
	23,15			34,52						
	23,96	23,95	0,04	36,73	36,84	0,26	12,89	3,02	0,12	0,17
Cp3r	23,91			37,14						
	23,98			36,66						
	24,58	24,58	0,09	36,54	35,57	0,30	10,99	1,11	0,46	0,53
Cp9r	24,67			34,87						
	24,49			35,29						
	24,54	24,56	0,11	35,23	34,66	0,80				
D10R	24,68			34,1						
	24,47									
	24,32	24,37	0,14	36,04	34,72	1,26				
D10R	24,26			33,53						
	24,52			34,58						
D10R		**24,46**	**0,15**		**34,34**	**0,34**	**9,88**			
	26,09	25,93	0,14	36,86	37,00	0,19	11,07	0,72	0,61	0,65
D8R	25,85			37,13						
	25,84			37,7						
	26,52	26,50	0,25	37,1	37,43	0,30	10,94	0,59	0,66	0,71
CP2	26,73			37,52						
	26,24			37,68						
	26,98	26,47	0,19	38,71	37,00	0,33	10,53	0,18	0,88	0,89
CP4	26,33			37,23						
	26,6			36,76						
	27,77	27,74	0,15	38,87	39,08	0,30	11,34	1,00	0,50	0,58
CP11	27,57			39,29						
	27,87									
	25,84	25,90	0,11	37,62	37,44	0,25	11,54	1,20	0,44	0,49
CP12	25,83			37,26						
	26,02			38,62						

	26,68	26,67	0,15		39,205	3,30					
D10R	26,52			41,54							
	26,81			36,87							
	24,96	25,2566667	0,26	36,77	37,66	0,35					
D10R	25,37			37,41							
	25,44			37,91							
D10R		**26,67**	**0,15**		**37,02**	**0,34**	**10,35**				
	23,84	23,85	0,12	37,24	37,86	0,01	14,01	0,69	0,62	0,65	
ET7	23,74			37,87							
	23,98			37,85							
	25,14	25,11	0,06	37,25	36,10	0,37	10,99	-2,33	5,04	3,85	
ET8	25,15			35,83							
	25,04			36,36							
	26,47	26,26	0,25	40,54	37,77	0,18	11,51	-1,81	3,51	2,96	
FO9	26,32			37,64							
	25,98			37,89							
	26,56	26,66	0,13	37,1	36,87	0,30	10,22	-3,10	8,59	6,29	
ET10	26,75			36,53							
	25,99			36,98							
	24,94	24,99	0,05	36,12	36,20	0,11	11,21	-2,11	4,32	3,38	
D1R	25,03			36,28							
	25,01										
	26,42	26,45	0,16	37,16	37,03	0,18	10,58	-2,74	6,67	5,07	
D3R	26,62			36,90							
	26,31										
	24,86	24,88	0,02	37,82	37,52	0,36	12,65	-0,67	1,59	1,47	
D4R	24,89			37,63							
	25,61			37,12							
	24,71	24,90	0,27	38,44	37,32	0,00	12,42	-0,90	1,86	1,68	
D7R	25,09			37,32							
	25,7			37,32							
	24,76	24,81	0,15	38,79	38,99	1,66					
D10R	24,69			40,75							
	24,97			37,44							
	25,83	25,61	0,33	38,26	38,13	0,19					
D10R	25,77			37,99							
	25,23			36,54							
D10R		**24,81**	**0,15**		**38,13**	**0,19**	**13,32**				

Statistical analyses were performed in R (R core Team, 2018) based on Livak fold change ($2^{-(\Delta\Delta CT)}$) values. Normality was tested using the Shapiro test and a histogram of all samples to observe their distribution pattern (Figure 3). The Shapiro test showed that MC1R gene expression values in adult stage do not follow a normal distribution (W=0.7611, p-value=0.0006) as is verified in histogram representation. Consequently, a non-parametric method, Mann-Whitney-Wilcoxon (MWW) test, was carried out to test whether melanic and non-melanic samples follow an identical distribution or, on the contrary, are independent. This test is also indicated when the sample size is less than 50, which is our case.

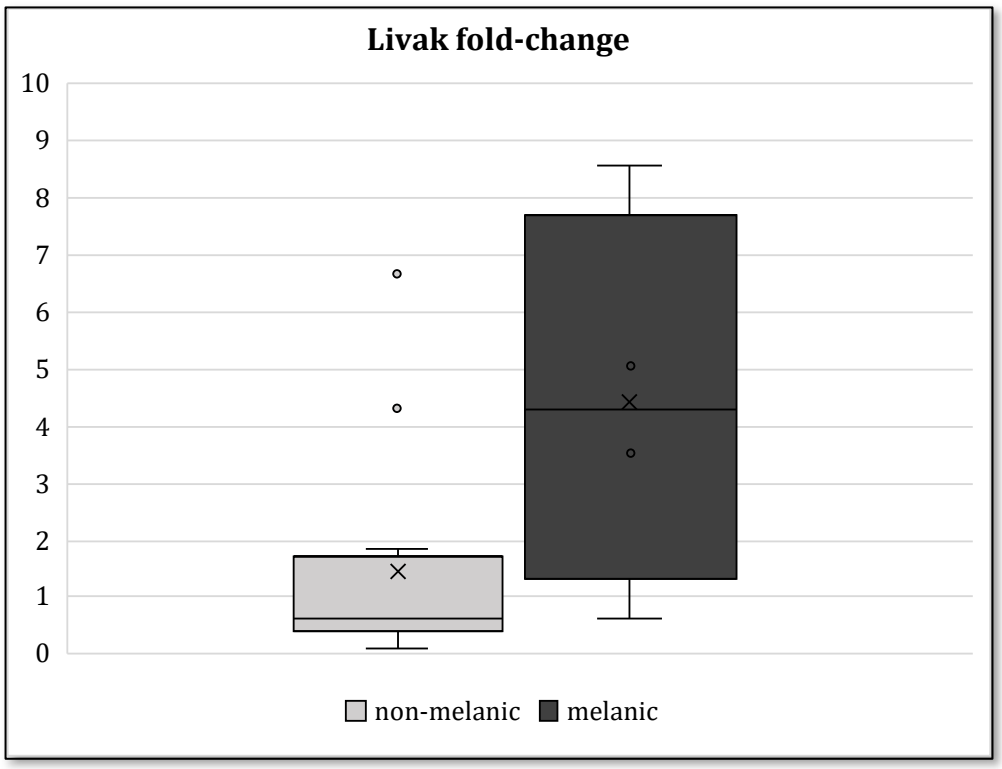


Figure 2. Quantifications of MC1R expression ($2^{-(\Delta\Delta CT)}$) in non-melanic (Dragonera and Cabrera harbour) and melanic (Esclatasang and Foradada) populations.

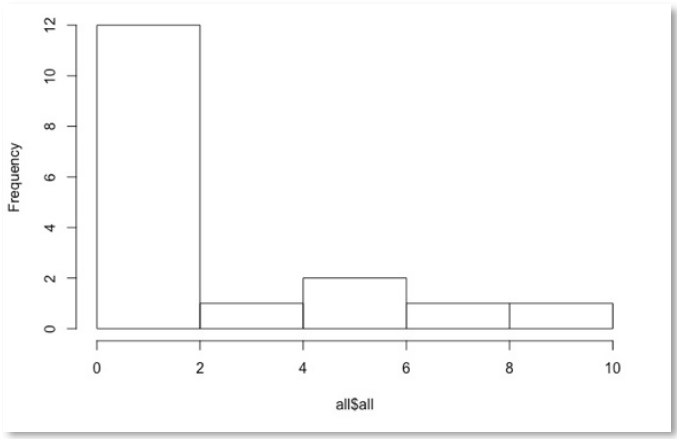


Figure 3. Histogram representation of all samples used in statistical analyses to test normality distribution.

Table 2. Resulting values of Mann-Whitney-Wilcoxon (MWW) statistical tests for different data comparison. Asterisk indicates significantly different comparison.

	MWW test
Melanic~non-melanic	0.0597
Dragonera~Cabrera	0.0734
Dragonera~melanic	0.3524
Cabrera~melanic	0.0242*
Dragonera+melanic~Cabrera	0.0136*

Mann-Whitney-Wilcoxon test results for melanic and non-melanic comparation were non-significant (p-value=0.05966), indicating that the MC1R expression values of the two population samples were not significatively different (Table 2). Other comparisons were performed with this test: a) Dragonera samples vs. Cabrera samples, b) Dragonera vs. melanic populations, c) Cabrera vs. melanic populations, and d) Dragonera+melanic populations vs. Cabrera samples. Only the comparisons between Cabrera vs. melanic forms (p-value=0.02424) and Dragonera+melanic samples vs. Cabrera samples (p-value=0.01357) were statistically significantly different.

These results are not in agreement with those found in a previous analysis included in Buades (2017), finding significant differences between melanic and non-melanic populations but with higher and more variable levels of MC1R expression in non-melanic samples. Although in this study non-significant differences were found between both groups of samples, there seems to be a tendency to higher and more variable values of melanic samples (Figure 2).

These contradictory results may be due to the fact that Buades (2017) used non-melanic samples from two different species (*P. lilfordi* and *P. pityusensis*). Either way, the low number of samples included in the present study could have had an effect on statistical analyses and is not enough to draw reliable conclusions.

Analysis of expression level of MC1R by RT-qPCR in different embryonic stages

Twelve (eight samples from Aire Island and four from Colom Island) (Table 3) of the total of 19 samples that presented good quality ratios in RNA extraction, amplified in RT-qPCR.

Table 3. Number of egg samples and different incubation time that present amplification in RT-PCR for non-melanic (Colom) and melanic (Aire) populations. Asterisk indicates the existence of duplicate samples.

Incubation (days)	0	8	11	14	16	17	19	25	28
Colom (eggs)	1	0	2*	1*					
Aire (eggs)	0				2*	1	1	1*	3

A standard curve based on the melanic sample from Aire Island (Menorca) for target (MC1R) and reference (ACTB) gene is shown in Figure 4. Efficiency values were 2.044 and 1.871, respectively.

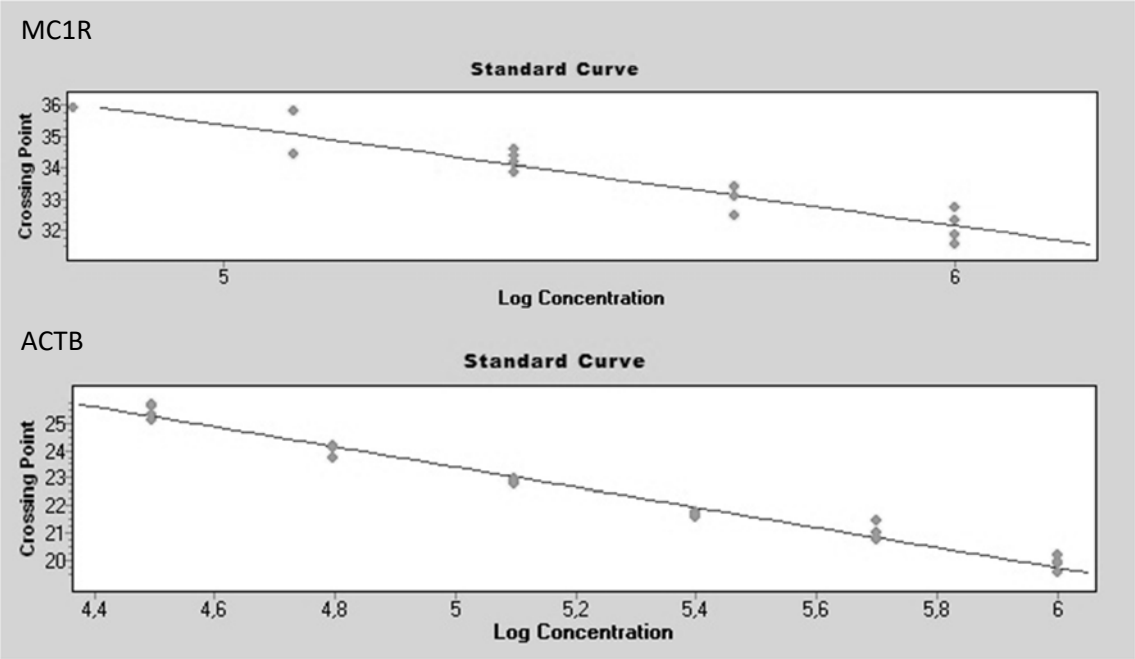


Figure 4. Standard curve for target (MC1R) and reference (ACTB) genes based on melanic sample from the Aire (Menorca) population for the analysis based on egg samples.

The fractional cycle numbers of threshold value (Ct) obtained from the RT-qPCR based on egg samples are indicated in Table 4. One of the three Ct replicate values was eliminated so as to obtain a standard deviation value of less than 0.30 (Taylor, 2009).

The ACTB gene showed a constitutive gene expression with a range of Ct values from 16.47-24.68, and with no significant differences between melanic and non-melanic samples. MC1R Ct values ranged from 30.62-36.10 in melanic individuals and from 33.77-36.94 in non-melanic samples (Table 1).

Table 4. Ct values, mean Ct, standard deviation (SD) for reference gene (ACTB) and target gene (MC1R) based on egg samples with different incubation times. Differences between target Ct and reference Ct (ΔCt) and differences between ΔCt of samples and ΔCt of calibrator sample (D10) are indicated. Livak and Pfaff values are also shown. Red values were eliminated to ensure a standard deviation < 0.30 (Ref) between the three replicates. Calibrator samples are highlighted in bold.

	ACTB ref			MC1R target			ΔCt	ΔΔCt		
Sample	**Ct**	**Mean Ct**	**SD**	**Ct**	**Mean Ct**	**SD**	**Ct(target)-Ct(ref)**	**ΔCt-ΔCt(calibrator)**	**Livak**	**Pfaff**
	24,65	24,68	0,06	36,68	36,94	0,25	12,26	0,22	0,86	0,72
Colom14_1	24,75			37,17						
	24,65			36,97						
	24,62	24,37	0,22	35,89	36,095	0,29	11,72	-0,31	1,24	1,08
Aire16_1	24,22			36,30						
	24,28									
	21,83	21,73	0,09	34,75	33,67	0,17	11,94	-0,10	1,07	1,17
Aire16_2	21,70			33,55						
	21,67			33,79						
		21,58	0,06	34,05	33,33	0,08	11,75	-0,29	1,22	1,35
Aire25_2	21,62			33,38						
	21,53			33,27						
	23,53	23,62	0,09	35,38	34,66	0,09	11,04	-1,00	2,00	1,88
Aire28_1	23,71			34,72						
	23,61			34,59						
		23,41	0,02	33,97	34,85	0,13	11,64	-0,39	1,31	1,27
Aire28_3	23,39			35,53						
	23,42			34,16						
	22,69	22,83	0,13	32,52	32,79	0,38	9,96	-2,08	4,23	4,37
Aire28_4	22,95			33,05						
	22,85			34,22						
	22,92	**22,68**	**0,22**	**34,90**	**34,72**	**0,26**	**12,035**			
Colom11_2	**22,64**			**34,53**						
	22,48			**35,95**						
	24,51	24,42	0,09		36,26	0,35	11,84	0,90	0,54	0,47
Colom0_1	24,33			36,51						
	24,42			36,01						
	21,37	21,47	0,09	34,99	35,00	0,22	13,53	2,59	0,17	0,18
Colom11_1	21,51			35,23						
	21,53			34,79						
	22,32	22,16	0,14	35,80	34,47	0,17	12,31	1,36	0,39	0,41
Colom11_1	22,09			34,35						
	22,08			34,59						
Colom11_2	**23,05**	**23,04**	**0,20**	**33,92**	**33,98**	**0,07**	**10,94**			

	23,23			**34,05**						
	22,83			**33,97**						
	16,38	16,47	0,13	30,95	30,76	0,35	14,29	1,04	0,49	0,68
Aire16_2	17,33			30,97						
	16,56			30,35						
	22,44	22,37	0,07	35,57	35,42	0,21	13,05	-0,20	1,15	0,98
Aire17_1	22,3									
	22,38			35,27						
	21,91	21,74	0,17	34,35	32,28	0,05	10,53	-2,71	6,56	6,24
Aire19_3	21,57			32,31						
	21,75			32,24						
	16,73	16,61	0,15	31,82	30,62	0,01	14,01	0,77	0,59	0,82
Aire25_2	16,65			30,61						
	16,44			30,63						
	19,84	20,59	0,15	35,46	35,70	0,33	15,11	1,87	0,27	0,26
Colom14_1	20,69			34,34						
	20,48			35,93						
	20,84	**20,52**	**0,28**	**33,75**	**33,77**	**0,02**	**13,25**			
Colom11_2	**20,31**			**32,51**						
	20,41			**33,78**						

RT-qPCR analysis of MC1R expression from non-melanic and melanic lizard populations based on different embryonic stages is shown in Figure 5. Fold changes ($2^{-(\Delta\Delta CT)}$) ranged from 0.49 to 6.56 in melanic samples, and from 0.17 to 0.86 in non-melanic individuals. Melanic samples showed a wider range of values than non-melanic samples. The mean for melanic samples was higher (1.99) than the non-melanic one (0.45). Similar results were found using the Livak & Schmittgen (2001) and Pfaff (2001) methods.

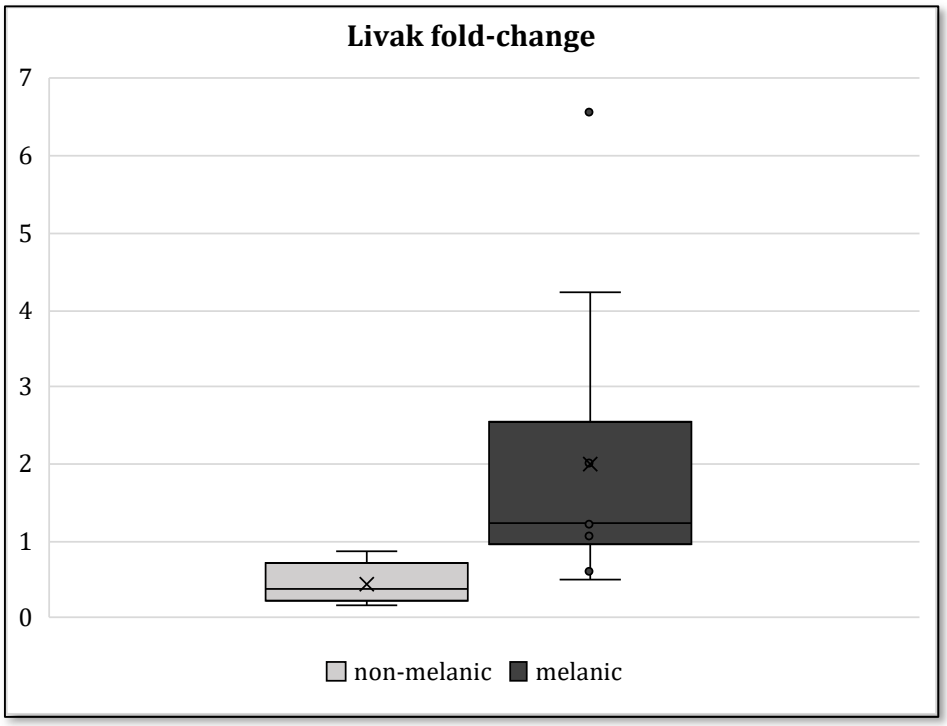


Figure 5. Quantifications of MC1R expression (Livak fold-change) in non-melanic (Colom) and melanic (Aire) populations.

The same statistical analyses previously performed with tail samples were carried out with egg samples. Distribution pattern of all samples are shown in Figure 6. The Normality Shapiro test indicated that gene expression values in embryonic stage do not follow a normal distribution (W=0.6823, p-value=0.0002) either. Consequently, the MWW test was also performed to test whether melanic and non-melanic sample values were significatively different.

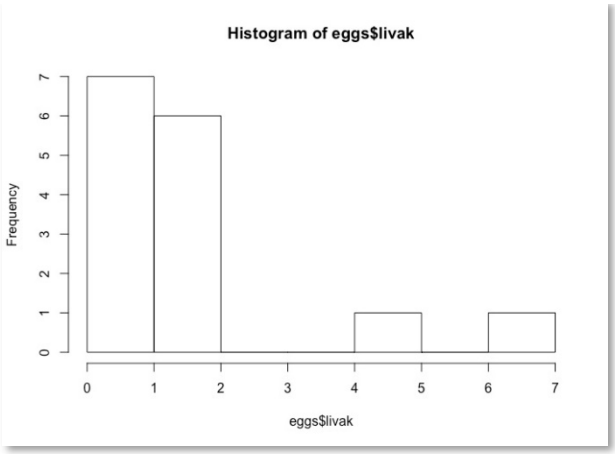


Figure 6. Histogram representation of all egg samples used in statistical analyses to test normality distribution.

The non-parametric test (MWW) indicated statically significant differences between melanic and non-melanic samples (p-value=0.0046) (Table 5) at embryonic stage. The test was also used to determine whether there were differences within melanic samples with equal or less than 16 days of incubation and samples with an incubation period higher than 16 days (Table 5), turning out to be non-significantly different. Even though the statistical test indicated that there are no statistically significant differences between the different stages of embryonic development, it would seem that MC1R expression tends to increase with time (Figure 7).

Table 5. Resulting values of Mann-Whitney-Wilcoxon statistical tests for different data comparisons based on egg samples.

	MWW test
melanic~non-melanic	0.0046*
melanic(d≤16)~melanic(d>16)	0.1833

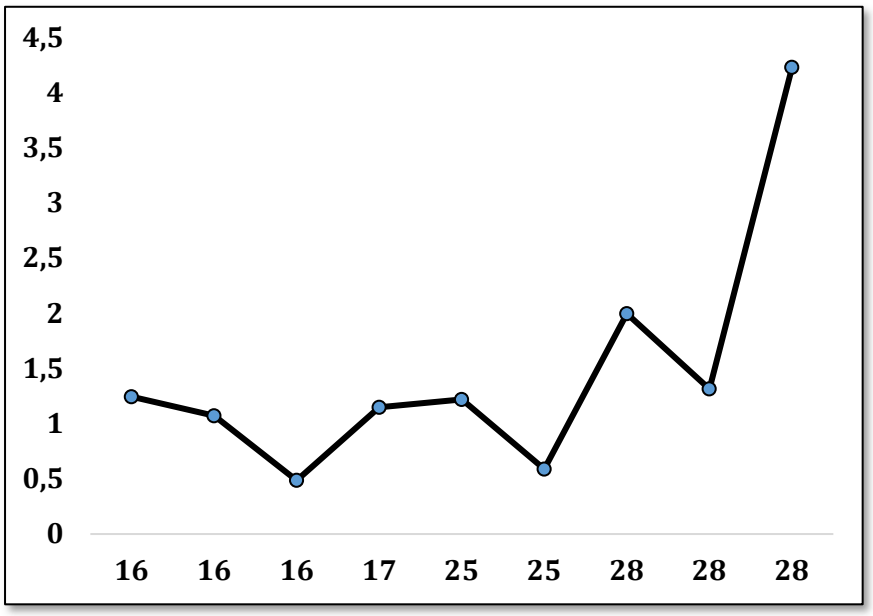


Figure 7. Graphic representation of MC1R expression (Livak fold-change) values for different embryonic stages for the melanic (Aire) population. In the graph, the outlier value (6.56) at 19 days of incubation was eliminated.

Difficulties encountered both in the maintenance of eggs in the terrarium until the later stages of development and in the RNA extraction due to their instability meant that the number of samples used in this study was diminished. More egg samples at each stage of development would be necessary to be able to infer reliable conclusions.

5

Discussion

Lizards have been considered model organisms for different ecological and evolutionary studies because they present several optimal characteristics, such as their broad geographical distribution and the wide range of habitats they inhabit, as well as the diverse morphologies and ecologies they present. In addition, lizards are normally abundant and their capture and manipulation in the field is usually easy to perform. Thus, there is vast knowledge concerning demographics, life history, and adaptive ecophysiology and ecomorphology in lizards. This has made these organisms a model for several evolutionary studies, from individual to community approaches on different temporal and spatial scales (e.g. Pianka & Vitt, 2003; Vitt & Caldwell, 2014, Camargo et al., 2010).

Mitochondrial DNA genes have been the most used markers to perform phylogenetic and phylogeographic studies in lizards, and in most other organisms (Zink & Barrowclough, 2008; Avise, 2009; Barrowclough & Zink, 2009). However, the increasing expansion of new markers (nuclear loci and genomic approaches), their cost reduction, and advances in data analyses have sped up the emergence of multilocus studies (e.g. Avise, 2000; Nielsen & Beaumont, 2009; Hickerson et al., 2010; Garrick et al., 2010; Arumugam et al., 2019), whilst simultaneously increasing the scope of these studies (Swenson, 2008; Hickerson et al., 2010; Sinervo et al., 2010; McCormack et al., 2013). Analyses based on a multilocus approach enable better precision in parameter estimation, such as population sizes or divergence times (Edwards & Beerli, 2000; Felsenstein, 2006; Heled & Drummond, 2008; Kuhner, 2008), and increase the robustness of species delimitation methods even if speciation events are very recent (Hickerson et al., 2006; Knowles & Carstens, 2007, Richards et al., 2007; Nielsen & Beaumont 2009; Templeton, 2009). The combination of species delimitation methods, phylogeography, and morphology, as well as the evaluation of the role of gene flow and natural selection across environmental gradients (Rosenblum, 2006) and biodiversity patterns (Leaché et al., 2007; Victoriano et al., 2008; Hurt et al., 2009; Moritz et al., 2009) contribute to the improvement of conservation strategies (Davis et al., 2008; Mateos et al., 2019; Santos et al., 2020).

As examples of multilocus studies carried out in different organisms, it is worth commenting on those of Leavitt et al., (2017), where the availability of nuclear markers led to a better understanding of the role of introgressive hybridization and mtDNA capture in confusing phylogenetic hypotheses based only on mtDNA in alligator genus *Elgaria*; or the study performed by Miralles et al., (2020), an extensive morphological and multilocus analysis in the taxonomy of *Acanthodactylus erythrurus*, revealing the existence of five species in the *Acanthodactylus* species complex, bringing more light to in this unresolved phylogeny. In addition, a comprehensive multilocus dataset of the gekkonid genus *Cyrtodactylus* on the island of Borneo was provided by Davis et al., (2020) to validate taxonomic status, evaluate species diversity, and clarify biogeographic patterns. In Vázquez-López et al., (2020) independent evolutionary lineages were discovered within *Euphonia affinis* based on the combined evidence of genetic, morphological, behavioural, and ecological data. The usefulness of a multilocus approach was also verified in *Pseudotrapelus* lizards (Tamar et al., 2019), where cryptic diversity was discovered within the genus. Phylogenetic inference, based on a wide sample dataset of multilocus genetic and morphological data among the *Japalura* genus, has clarified problematic taxonomy and unknown phylogenetic relationships (Wang et al., 2019b). Some species relationships among skinks genera (*Lepidothyris*, *Lygosoma* and *Mochlus*), however, are still not well-defined. Multilocus data analyses provide a better reflection of the evolutionary history of these reptiles (Freitas et al., 2019).

Multilocus approach and influence in conservation strategies

In this thesis, the usefulness of a multilocus approach were tested on the *P. hispanicus* species complex and on currently recognized subspecies of *P. lilfordi*.

The study based on the *P. hispanicus* species complex has shed some light on the phylogeographical pattern and phylogenetic relationships among the evolutionary lineages making up this complex. Three principal clades had been previously found in this complex: western (*P. carbonelli*, *P. virescens*, *P. guadarramae*, and *P. bocagei*); south-eastern (*P. vaucheri*, *P. hispanicus* (Valencia), and *P. hispanicus* (Albacete/Murcia)); and north-eastern including *P. liolepis* and *P. hispanicus* (Galera) (Kaliontzopoulou et al., 2011).

Specifically, our study focused on the region of the SE Iberian Peninsula where different lineages present an overlapped distribution and, despite the numerous studies based on this species complex (Harris & Sa-Sousa, 2002; Pinho et al., 2006, 2007, 2008; Renoult et al., 2009, 2010; Kaliontzopoulou et al., 2011; Geniez et al., 2014), they were still not well defined. Both morphological and multilocus genetic approaches used in this study made it possible to identify three differentiated groups in this region: *P. galerai* sp. nov, *P. hispanicus sensu stricto* (Albacete/Murcia), and *P. hispanicus* (Valencia lineage). In this study, *P. galerai* sp. nov has achieved full species status and the nominal form of the complex (*P. hispanicus sensu stricto*) was redefined, with the Albacete/Murcia lineage becoming the nominal type, as it is the population located at the closest point of the restricted-type locality of Monteagudo (Geniez et al., 2007) and it presents more similar morphological traits than those proposed by Geniez et al. (2007) for the nominal taxon. Contrary to previous findings (Kaliontzopoulou et al., 2011), in our study the north-eastern form *P. liolepis* forms a clade without Galera lineage and with low support, which could be explained because the overall distribution of this lineage is incomplete.

The geological history of the SE region of the Iberian Peninsula also explains the isolation of *P. galerai* sp. nov. clade, located in the south of the Betic cordillera, since during the middle Miocene (Serravallian/Tortonian) the Betic corridor connected the Mediterranean Sea and the Atlantic Ocean separating the Iberian Peninsula at the Betic cordillera. The definitive closure of this corridor took place during the Messinian, provoking the connection between Africa and the Iberian Peninsula (Krijgsman et al., 1996, 2000, 2018) and bringing about the divergence of several groups of organisms (Carranza et al., 2004b; Busack et al., 2005; Pinho et al., 2006; Albert et al., 2007; Paulo et al., 2008; Kaliontzopoulou et al., 2011). In the Messinian period, the divergence time of the other SE lineages (Valencia and Albacete/Murcia) have been dated, explaining the closer position of these lineages with the North African clade than with other Iberian Peninsula lineages.

Although both phylogenetic and species delimitation analyses, corroborated with multiple methodological approaches and using different datasets (mtDNA, nuclear loci or combined data), showed high supports for the different SE *Podarcis* groups, there were some limitations that could be clarified by further analyses (Amor et al., 2019; Linck et al., 2019; Quattrini et al., 2019). For instance, the lack of information regarding the global distribution of *P. liolepis* and Valencia lineage, which would contribute to obtaining a more complete picture of the distribution of the *Podarcis* genus in the Iberian Peninsula. In addition, it would be interesting to perform a global morphological study in order to clarify the high diversity present in this species complex (De Jesus et al., 2019; Dömel et al., 2019).

Another region with an unclear evolutionary history is the Columbretes archipelago, inhabited by *Podarcis* lizard that first was considered part of the *P. hispanicus* complex as *P. h. atrata* (Bauer & Günther, 1995) and later as a new endemic species named *P. atrata* (Castilla et al., 1998a). Currently, it is considered conspecific with *P. liolepis* (Harris et al., 2002, Harris & Sá-Sousa, 2002, Pinho, 2006, 2007, 2008, Renoult et al., 2010, Kaliontzopoulou et al., 2011), which occupied the northeast of the Iberian Peninsula. Castilla et al. (1998) used universal primers (Kocher et al., 1989) that probably caused a non-specific amplification of mtDNA CYTB gene, but the co-amplification of nuclear mitochondrial DNA segments (NUMTs). The high genetic diversity found between *Podarcis* forms of Columbretes Islands and from the mainland of the Iberian Peninsula could be due to this unspecific amplification. However, it may also be because Castilla et al. (1998a) compared Columbretes samples with populations from one of the *P. hispanicus* lineages (*P. h. sensu stricto*, *P. hispanicus* (Valencia) or *P. galerai* sp. nov.), not yet described in the SE region of the Iberian Peninsula, rather than *P. liolepis*.

In our study, the evolutionary origin of these insular lizard populations was explored and supported by geological events. The belonging of the *Podarcis* from Columbretes Islands to *P. liolepis* form from the mainland of the Iberian Peninsula was corroborated, but additionally our findings located the *P. liolepis* form that originated the insular lizards specifically in Peñagolosa situated 50 km away from the Columbretes Islands. Current results also point out that they may have diverged at 1.77 Ma, coinciding with a period with several sea level fluctuations (Emig & Geistdoerfer, 2004) during which the Columbretes Islands and mainland Iberian Peninsula could have been connected. In addition, our study reveals that these insular populations could have suffered several events of decrease in diversity (bottlenecks) and/or expansion in their demographic history.

The great divergence within *P. liolepis* forms inhabiting Iberian Peninsula hinder the establishment of phylogenetic relationships between them and the other species complex forms. Obtaining a more complete distribution of *P. liolepis* would help to better understand the phylogenetic and phylogeographic relationships between insular and mainland *Podarcis* populations. Since the divergence time of the Columbretes Islands is very recent (1.77 Ma), it may be interesting to use genomic approaches so as to gain a better understanding of their evolutionary history, determine the effects of the growth-decline events in the population, and clarify their conservation status (Brüniche-Olsen et al., 2019; Wee et al., 2019; Williams et al., 2020).

Regarding *P. lilfordi* (Günter, 1874), this endemic species inhabits the Gymnesian Islands (Balearic archipelago) and includes 24 subspecies located in 42 geographical sites. These locations are distributed in Mallorca (10 islands), Menorca (16 islands), and the Cabrera archipelago (Pérez-Mellado, 2009; Terrasa et al., 2009a; Pérez-Cembranos et al., 2020). These populations differ in morphological, genetic, ecological, and ethological characteristics and this variability does not correspond with the number of subspecies currently described. Based on multilocus and morphological datasets, the study performed in this thesis concludes that the definition of ESUs (Moritz, 1994, 2002; Waples, 1995; Karl & Bowen, 1999) as opposed to subspecies is better to recognize genetic and morphological diversity, to ensure the evolutionary process, and to take into account in conservation strategies (Crandall et al., 2000; Fraser & Bernatchez, 2001). The same problematic in terms of species and subspecies boundaries and delimiting conservation units was found by Phillimore et al., (2008) in an island endemic bird (*Zosterops flavifrons*) population, and by Cooper et al., (2020) in *Isoodon* taxa when observing discordance between phylogeographic and population genetic analyses. Mussmann et al., (2020) also highlighted the importance of a multifaceted delimitation of conservation units.

The geographical isolation that these microinsular populations suffer means that each population presents an evolutionarily independent history linked to different ecological conditions, remarkably variable population sizes and availability of trophic resources, besides the presence/absence of competitors and predators. These circumstances have led to different adaptive responses, in some cases unique to each population, that are translated into ecological, morphological, and ethological characteristics (Karl & Bowen, 1999; Manceau et al., 1999; Martín et al., 2017; Barbosa et al., 2018).

Genome-wide studies in model and non-model organisms has had a considerable impact on evolutionary biology (Hohenhole et al., 2010; Nosil et al., 2012; Guo et al., 2015), since the proportion of genome studied has increased, thereby enabling a more robust evaluation of population divergence processes and the identification of genomic regions of interest (Rincon-Sandoval et al., 2019; Ottenburghs et al., 2019; Dufresnes et al., 2020; Ewart et al., 2020; Lim et al., 2020). In reptiles, several studies use a genomic approach to understand population divergence (Brown et al., 2016; Jin & Brown, 2019; Reynolds et al., 2020), clarify phylogeography (Leaché et al., 2020), and help in the identification of conservation units (Sovic et al., 2016; Vargas-Ramirez et al., 2020).

The genomic approach carried out in this thesis revealed a clear genetic population structure among *P. lilfordi* populations and validated the high divergence between Menorca and Mallorca+Cabrera populations (Terrasa et al., 2009a; Brown et al., 2008). It also indicated a closer position of the Colomer islet to Mallorcan populations than to Cabrera forms, as was found previously, supporting the hypothesis that Colomer could be a relict population of the ancient populations now extinct from the main island of Mallorca suggested in Terrasa et al. (2009a). This study also corroborated that population size plays a remarkable role in genomic divergence, since the smallest populations showed the most divergent positions in all genetic analyses. These results point out that patterns of genetic diversity among lizard populations on islands are largely due to limited dispersal and that genetic drift has driven divergence (Brown et al., 2016; Li et al., 2019; Ciofi et al. 1999; Malone et al. 2003; Nichols & Freeman, 2004; MacAvoy et al., 2007; Debortoli et al., 2020; Isshiki et al., 2020; Oliveira et al., 2020; Venables et al., 2020). Genome-wide study reinforced the value of considering each *P. lilfordi* population as independent evolutionarily units (ESUs), due to their geographical isolation and their characteristically ecological features.

Adaptive divergence

Genome-wide studies also enable putative genomic regions under selection to be identified and the effect of adaptive divergence to be evaluated (Nosil, 2004; Brown et al., 2016; Raeymaekers et al., 2017; Rodríguez et al., 2017b).

In the genomic study of *P. lilfordi* included in this thesis, based on ddRADseqs, the evidence of adaptive divergence among lizard populations was highlighted. Almost 2% of total SNPs were candidates to be under selection and these loci seem to be related to adaptations like tail regeneration, reproduction, lipid metabolism, or circadian rhythm, among other functions. Our results are in agreement with different studies showing the connection between genetic divergence and geographical distribution of species, patterns of colonization, and/or landscape gradients (Benestan et al., 2016; Campbell-Staton et al., 2016; Rodríguez et al., 2017b; Prates et al., 2018). In our study, environmental variables appear to be an important driver of divergence between lizard populations, according to genomic patterns.

The highest number of associated SNPs was related to levels of predation and human pressure in the lizard habitat. These associated SNPs were involved in diverse functions, with those related to feeding and locomotory behaviour standing out. The influence of the remaining predictors included in the analysis, such as melanism, biotic capacity of islands, presence of rats, or the existence of breeding colonies of gulls, were negligible. Some behavioural and physiological differences between populations might be related to differences in predation and human pressures (Vervust et al., 2007; Ingley et al., 2014; Kang et al., 2017; Marques et al., 2018; Belk et al., 2020), as in the case of escape behaviour in lizard populations with or without terrestrial predators. In the Balearic lizard, there is a significant effect of predation pressure on flight initiation distance, distance fled, or hiding time (Cooper et al., 2009; 2010; Cooper & Pérez-Mellado, 2010, 2012), that is on the willingness of organisms to take risks.

The Balearic lizard evolved in an environment free of terrestrial predators for more than five million years (Bover et al., 2008). The arrival of humans in the Balearic Islands brought about the introduction of several allochthonous predators (Bover et al., 2008, 2014; Pérez-Mellado, 2009), consequently, *P. lilfordi* had to face strong selective pressure from human and foreign predators from the Holocene. The interesting result is that this selective factor, acting only over the last 5,000 years, has had a strong and detectable effect on the genomic structure of these populations. Some studies (Vervust et al., 2007; Marques et al., 2018) in other organisms provide examples of how environmental factors may produce responses in behaviour and morphology on a surprisingly small spatial and temporal scale, as in the *P. lilfordi* situation.

Despite the minor influence that melanism presents in *P. lilfordi* populations as a predictor of adaptive divergence in the genome-wide analyses performed in this thesis, melanism has been the focus of several studies on evolutionary adaptation (Cox & John-Alder, 2005; Janse van Rensburg et al., 2009; Alho et al., 2010). The role of melanism has been related to several different adaptive functions such as sexual selection, thermoregulation, UV protection, immune response, stress resistance, or response to predation risk (Nosil et al., 2004; Ducrest et al., 2008; Dubovskiy et al., 2013; Fedorka et al., 2013; Fulgione et al., 2014; Reguera et al., 2014; Azócar et al., 2016; Bliard et al., 2020).

No relationships were found between *P. lilfordi* populations with different pigmentation patterns (melanic and non-melanic) and their geographical situation or genetic divergence in both multilocus and genomic approaches carried out in the current thesis. Even though melanic colouration seems to confer relevant adaptive advantages to its holders (Wilson et al., 2001; Vences et al., 2002; Callaghan et al., 2004; Calbó et al., 2005; Clusella-Trullas et al., 2007; Ducrest et al., 2008; Vroonen et al., 2012; Fedorka et al., 2013; Reguera et al., 2014) and consequently appears in different lineages of the Balearic lizard, it does not seem to play a key role in driving divergence between lizard populations, as indicated in the genomic study.

The MC1R gene expression analyses in different development stages of melanic and non-melanic *P. lilfordi* populations included in this thesis gave inconclusive results. In adult stage, non-significant differences were found between melanic and non-melanic populations. Only the analysis based on egg samples with different development stages showed that melanic and non-melanic populations were significantly different in MC1R expression. However, no significant differences were found between the different stages of embryonic development (samples with 16 days or less vs. samples with more than 16 days of incubation) in melanic population.

Our results in adult stages do not agree with the study of Fulgione et al. (2015) in *Podarcis siculus*, where MC1R expression was higher in dark specimens, or in Japanese quails (Zhang et al., 2013a), with similar results, but it does agree with several other studies where no variation was found in gene expression (Han et al., 2012; Peñagaricano et al., 2012; Bradley et al., 2013). The significant high expression of MC1R observed in melanic populations compared to non-melanic population during embryonic development was also found in hyperpigmented individuals in Japanese quail embryos (Gluckman & Mundy, 2017; Li et al., 2018).

Overall, the gene expression analysis was based on a very low number of samples and specifically in egg samples not all stages of development were represented, so consequently the results may be affected. The low number of samples was due to problems in RNA extraction since RNA is very sensitive. The problem with egg samples lies in the difficulty of maintaining the incubation of the eggs until the last stages of development, especially in Colom Island samples. Better, wider sampling would be necessary in order to obtain more reliable results.

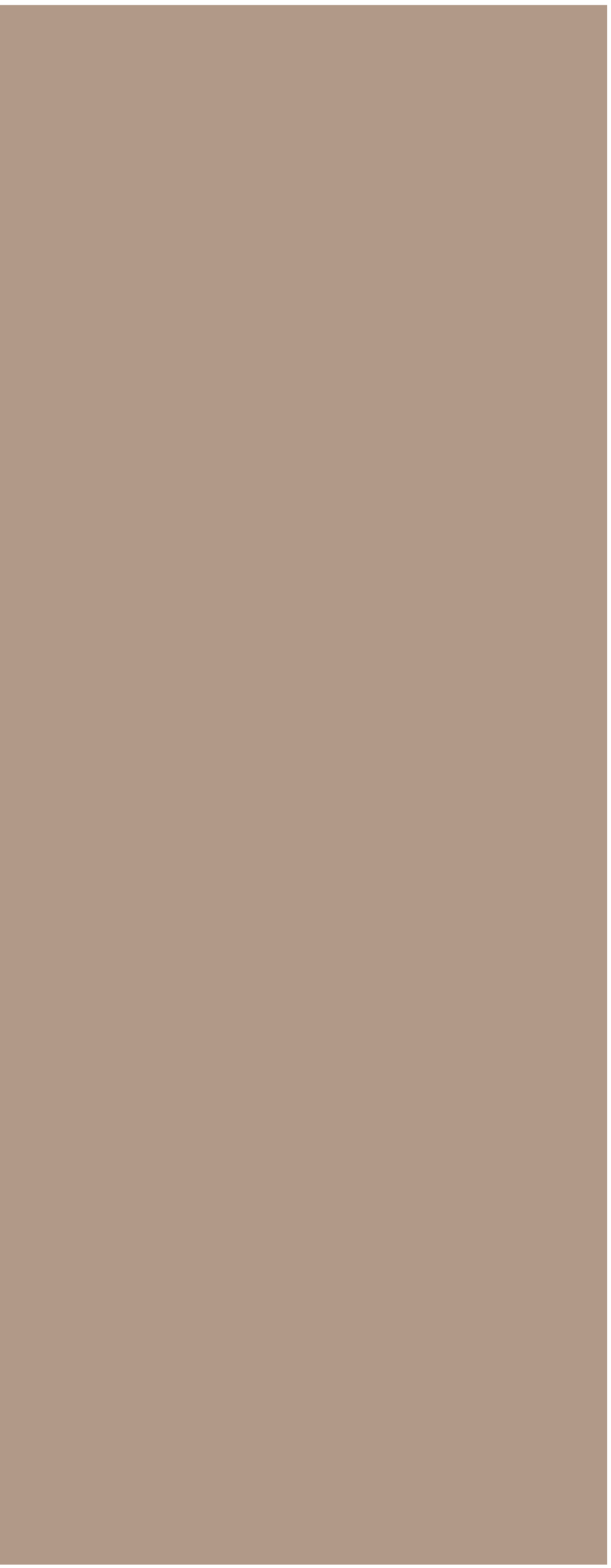
In summary, the importance of using more and different genetic information (mtDNA, nuclear markers and/or genomic approaches) and increasing the number of individuals and locations sampled so as to obtain a better distribution of genetic lineages should be highlighted. The results found in the *P. hispanicus* species complex enable a deeper phylogenetic and phylogeographical analysis that has shed light on some points of controversy found in previous studies. The combined phylogenetic and morphological knowledge achieved in these studies will allow for better taxonomic decisions to be made in some of the extraordinary diverse mitochondrial lineages of the Iberian *Podarcis*, such as SE lineages and Columbretes Islands populations. We must not forget that the evolutionary history, systematics, and biogeography of the Iberian *Podarcis* is far from complete and the current picture of this species complex can vary with future deeper analyses, especially in *P. liolepis* and *P. hispanicus* (Valencia lineage).

The uniqueness of *P. lilfordi* populations indicates that the recognition of each population as ESUs is better than the current classification by subspecies. Consequently, each of the Balearic lizard populations is fully relevant for their conservation and the future process of evolution of the Balearic lizard will be preserved. In addition, genome-wide methodology has enabled the increase of knowledge regarding the processes related to genetic divergence and the identification of genomic regions of interest. Overall, our results reveal that both neutral processes, associated with isolation and small population size, and selective factors, related to environmental patterns (specifically human pressure and level of predation) have played a role in shaping divergence between Balearic lizard populations. The genomic approach reinforced the value of considering each *P. lilfordi* population as independent evolutionary units, due to their geographical isolation and their ecological features.

Finally, more studies are needed to evaluate the effect of selection and adaptation on the evolutionary history of the different *P. lilfordi* populations, particularly to detect which factors or selective pressures have played a key role in the colouration divergence between the different insular populations.

6

Conclusions



1. A multilocus approach with large sampling enabled the phylogenetic relationships in the southeast (SE) region of the *P. hispanicus* complex of the Iberian Peninsula and in the Columbretes archipelago to be clarified. In the SE region, three different lineages were defined based on mtDNA, nuclear gene, and morphological data: the nominal taxon (*P. hispanicus sensu stricto*), *P. hispanicus* (Valencia lineage) and *P. galerai* sp. nov.

2. *Podarcis galerai* sp. nov. achieved full species status supported by phylogenetic and geological history. Divergence time between *P. galerai* sp. nov. and the other SE lineages was dated at 12.58 Ma, coinciding with the separation of the Betic Corridor.

3. *Podarcis hispanicus sensu stricto* was identified as the nominal taxon since it is situated at the closest point to the restricted-type location (Monteagudo, Murcia) and presents the morphological traits that are most similar to those described in Geniez et al., (2007) for the nominal taxon. *Podarcis hispanicus* (Valencia lineage) needs more sampling and a better definition of their distribution in order to fully establish its taxonomic status.

4. The *Podarcis* from the Columbretes archipelago presented low genetic variability, and the effects of expansion events and/or decrease in population size (bottlenecks) were detected. Phylogenetic results corroborated that the *Podarcis* populations that inhabit the Columbretes archipelago are conspecific with *P. liolepis*, specifically related to those in the Peñagolosa region located in Castellon, 50 km from the archipelago. The divergence time between the insular and mainland forms was dated at 1.77 Ma, coinciding with a period of several sea level fluctuations.

5. Regarding Balearic endemic lizard (*P. lilfordi*) populations, variability in ecological and morphological traits, as well as at the genetic level, points out the need to consider the different insular populations as ESUs, and not as subspecies. Genome-wide analyses also corroborated the uniqueness of *P. lilfordi* populations.

6. The genomic approach highlighted the influence of genetic drift in populations with a small size and geographic isolation, and the role of adaptive selection in shaping the genetic divergence of the *P. lilfordi* species. Human pressure and predation were shown to be the most important environmental variables driving adaptive divergence between these insular populations.

7. Non-significant differences in MC1R expression were found between melanic and non-melanic *P. lilfordi* populations at adult stages, but significatively higher expression values were found in melanic populations at embryonic stages. It seems that there is a tendency for MC1R gene expression to increase with egg incubation times in melanic populations, although more data would be needed to draw conclusions.

7

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