



# Colonization history of Mallorca Island by the European rabbit, *Oryctolagus cuniculus*, and the Iberian hare, *Lepus granatensis* (Lagomorpha: Leporidae)

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The Mediterranean islands have a long history of human-mediated introductions resulting in frequent replacements of their fauna and flora. Although these histories are sometimes well documented or may be inferred from paleontological studies, the use of phylogenetic and population genetic reconstruction methods provides a complementary perspective for answering questions related to the history of insular species. In the present study, we infer the colonization history of Mallorca (Balearic Islands) by the European rabbit (*Oryctolagus cuniculus*) and the Iberian hare (*Lepus granatensis*) using sequence variation of the mitochondrial DNA control region from continental and insular specimens (total of 489 sequences). Additionally, the taxonomic identity of Mallorcan *L. granatensis* was confirmed using a diagnostic nuclear marker. For both Mallorcan rabbits and hares, genetic diversity was comparable to the continental populations, suggesting the introduction of multiple lineages. Two Mallorcan haplogroups were found in hares, which likely correspond to two introduction events. Rabbits from Mallorca were identified as belonging to the subspecies *Oryctolagus cuniculus cuniculus*, and may have been originated both from Iberian and French populations. The molecular estimates of the timing of the colonization events of the Mallorcan lagomorphs are consistent with human-mediated introductions by early settlers on the islands. © 2014 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2014, 111, 748–760.

**ADDITIONAL KEYWORDS:** control region – introductions – lagomorphs – Mediterranean – peninsula – phylogeny – phylogeography.

## INTRODUCTION

Islands are considered as natural laboratories for the study of evolution, particularly with respect to colonizations and extinctions (Lomolino, 2000). Understanding the framework of this process and distinguishing natural from human-mediated introductions can often be challenging because of a lack of

historical records of introductions. The advent of molecular genetics has provided invaluable contributions for better understanding the native or alien nature of fauna and flora populating islands, as well as for clarifying the impact of the founder event in the genetic diversity of these taxa (Sly *et al.*, 2011).

The Balearic Islands comprise an archipelago located in front of the eastern coast of the Iberian Peninsula. As a result of their geographical position in the Western Mediterranean Sea, they were of great

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importance for ancient navigation for at least 4000 years (Bellard, 1995). These islands have been settled throughout their history by various civilizations, such as the Punic and Romans (Alcover, 2008), which have left important marks in the biotic and abiotic features of the territory (Uerpmann, 1971; Alcover, 1982). Contradicting previous suggestions that the colonization of the Balearics by extant mammals was very old, Alcover (1982) proposed that most of the fauna presently occurring in the archipelago was introduced into the islands only recently by man. This idea is supported by the absence of paleontological findings representative of any presently living mammal of the Balearics older than the Holocene (Adrover, 1966). According to Alcover (1982) and Bover, Quintana & Alcover (2008), apart from bats and birds, only three terrestrial mammals (the Balearic Cave Goat, *Myotragus balearicus*, Bate 1909; the Mallorcan giant dormouse, *Hypnomys morphaeus*, Bate 1918; and the Balearic shrew, *Nesiotites hidalgo* Bate 1944), a lizard (*Podarcis lilfordi* Günther 1874), and one amphibian (*Alytes muletensis* Sanchíz and Adrover 1977) existed in Mallorca until the Holocene. Only with the arrival of humans did other nonflying mammals colonize the island.

The first known written record of the presence of rabbits in the Balearic Islands reports a rabbit plague in the Mallorca and Menorca islands by the time of the emperor Augustus Caesar (approximately 2000 BP), which can be found in Pliny's the Elder book, *Naturalis Historia*. Pliny based his writings on other books dating from that time and on stories told by others. Thus, the information in his book may not be reliable. Rabbits also have mythological importance in Mallorca and legends of the existence of a rabbit-hare like species, locally named 'esquirol', persist until the present. However, nothing can be interpreted from these writings with respect to the introduction of lagomorphs (hares and rabbits) into the island, which could date back to the arrival of the Romans (who conquered the island approximately 2100 BP) or to the time of the Talayotic culture (settlers that lived on the island before the Romans). Reumer & Sanders (1984), based on palaeontological data, suggested that rabbits were present in Menorca ever since 3350–3250 BP, although this date is tentative because no radiocarbon dating was performed. Regardless of the age of the first introductions of lagomorphs into Mallorca, anecdotal evidence suggests that more recent introductions for hunting purposes are known to have occurred, and there is an original breed of rabbits recognized in the Balearic islands (named 'Conill pagès d'Eivissa').

Based on morphological data, Palacios & Fernández (1992) described Mallorcan hares as a subspecies of the Iberian hare (*Lepus granatensis* Rosenhauer

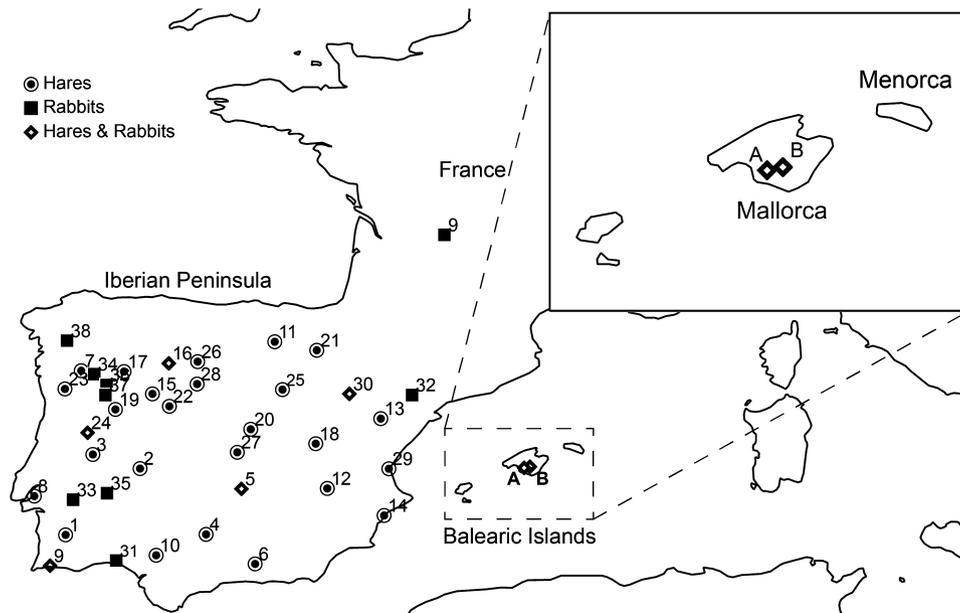
1856), *Lepus granatensis solisi*. In the mainland, this species has a broad distribution across the Iberian Peninsula, except in the north-eastern region where it is replaced by *Lepus europaeus* Pallas 1778, and in the Cantabrian mountain chain where *Lepus castroviejo* Palacios 1977 is found instead (Alves *et al.*, 2003). Rabbits are classified in genus *Oryctolagus*, which includes a single species (*Oryctolagus cuniculus* Linnaeus 1758) that originated in the Iberian Peninsula and naturally colonized Central Europe. Two subspecies are recognized: *Oryctolagus cuniculus algirus* Loche 1858, distributed in the south-western part of Iberia, and *Oryctolagus cuniculus cuniculus* Linnaeus 1758, present in the north-east of Iberia, Europe, and several other locations around the world where it has been introduced by man, such as Australia and South America (Branco, Ferrand & Monnerot, 2000; Ferrand & Branco, 2007). *Oryctolagus cuniculus algirus* is the rabbit subspecies that is assumed to inhabit the Balearic Islands (Gibb, 1990).

In the present study, the history of colonization of Mallorca by hares and rabbits, as well as their likely taxonomic status, was inferred using molecular data. The results were compared with historical documentation and previous studies. We aimed to contribute to a better understanding of the history of colonization of Mallorca by tracing the origins of Mallorcan hares and rabbits and disentangling hypotheses of human-mediated or natural colonizations.

## MATERIAL AND METHODS

### SAMPLING AND LABORATORY PROCEDURES

Tissue samples of 25 wild rabbits and 25 hares, mostly from road kills, were collected by Autonomic Environment agents around the Mallorcan localities of Lluçmajor and Porreres (Fig. 1). DNA from ear and muscle tissue was extracted using Genomic DNA Tissue Kit (EasySpin) in accordance with the manufacturer's instructions. A fragment of the mitochondrial (mt)DNA control region was amplified by the polymerase chain reaction (PCR) using primers LCRSEQ (5'-CACCATCAGCACCCAAAG-3') (Melo-Ferreira *et al.*, 2007) and LEPD2H (5'-ATTT AAGAGGAACGTGTGGG-3') (Pierpaoli *et al.*, 1999), both for rabbits and hares. In addition, DNA was extracted from 42 other wild rabbits collected from several locations in the Iberian Peninsula (Fig. 1; see also Supporting information, Table S1). A similar mtDNA control region fragment was amplified in these samples using primers Pro1 (5'-CCACCATCAGCAC CCAAAGCT-3') and NC4 (5'-ATGGCCCTGAGGTAAG AACC-3') (Mougel, 1997; Branco *et al.*, 2002). Cleaning of PCR products was performed with ExoSap-IT



**Figure 1.** Map of Western Europe showing the location of the island of Mallorca and sampling locations on this Island and on the European mainland. Detailed information on populations' codes is provided in the Supporting information (Table S1).

(according to the USB protocol) and then purified using Sephadex G-50 Medium (GE Healthcare) in CENTRI-SEP columns before sequencing. Sequencing was performed using the BigDye Terminator cycle sequencing protocol and using the LCRSEQ and NC5 (5'-CTTTAATAAACTCAAGTACTTC-3') (Mougel, 1997; Branco *et al.*, 2002) primers.

As a result of the documented mtDNA introgression in hares (Alves *et al.*, 2003, 2008; Melo-Ferreira *et al.*, 2005, 2007) and the subsequent inaccuracy of using mtDNA alone for species identification (Alves *et al.*, 2006), a diagnostic nuclear marker (UCP2; uncoupling protein 2) was amplified in all hare samples using primers LUCP2e6F1 (5'-TGCCGAGCTGGTCA CCTAT-3') and LUCP2i67R1 (5'-GACTTCCACTGA GGCCCTAAAA-3') (Melo-Ferreira *et al.*, 2009).

#### STATISTICAL ANALYSIS

The sequence dataset of both rabbits and hares was complemented with the inclusion of 28 rabbit and 429 hare sequences from the mainland recovered from GenBank (for GenBank accession numbers of Iberian hare and European rabbit sequences, see the Supporting information, Table S1). The sequences of the mtDNA control region of Mallorcan hares and rabbits were first aligned against a dataset of sequences known to be representatives of the major lineages of these species in the mainland (for hares, 10 representatives of the native and 10 of the introgressed lineage were used and, for rabbits, 10 representatives

of each subspecies were included; see Supporting information, Table S1), using CLUSTALW multiple alignment implemented in BIOEDIT, version 7.0.5.3 (Hall, 1999). The phylogenetic reconstruction was performed in MEGA, version 5 (Tamura *et al.*, 2011), using the maximum likelihood (ML) and Neighbour-joining (NJ) methods under the general time reversible and Hasegawa–Kishino–Yano mutation models with gamma distributed site heterogeneity rate (applied to hares and rabbits datasets, respectively) as determined in JMODELTEST, version 0.1 (Guindon & Gascuel, 2003; Posada, 2008). Branch support was assessed using 500 bootstrap replicates. In the case of hares, this allowed determination of whether the mtDNA lineage of the Mallorcan specimens was the native lineage or that introgressed from *Lepus timidus* and, in the latter case, to which sublineage it belonged (Melo-Ferreira *et al.*, 2007). For rabbits, the phylogenetic reconstruction allowed determination of whether Mallorca rabbits belonged to mtDNA clade A (*O. c. algirus*) or clade B (*O. c. cuniculus*) (Branco *et al.*, 2002). The relationships among Mallorcan and continental haplotypes were also examined using median-joining networks (Bandelt, Forster & Röhl, 1999) with maximum parsimony optimization (Polzin & Daneschmand, 2003), produced in NETWORK, version 4.5.1.6 (<http://www.fluxus-engineering.com>) in an extended dataset combining the mtDNA-CR sequences produced in the present study and previously published ones: 429 *L. granatensis* specimens from the Iberian Peninsula

(Melo-Ferreira *et al.*, 2007, 2011) and 28 *Oryctolagus cuniculus* from France (GenBank Accession numbers in the Supporting information, Table S1), for a total of 60 rabbit sequences from the European mainland.

Nucleotide diversity ( $\pi$ ; Nei, 1987), the proportion of segregating sites in a sample ( $\theta_S$ ; Watterson, 1975) and haplotype diversity ( $h$ ) were estimated in ARLEQUIN, version 3.11 (Excoffier, Laval & Schneider, 2005) using the extended dataset. The mismatch distributions were calculated using the same software, and fitted to the Sudden Expansion Model (Rogers & Harpending, 1992). This distribution is usually multimodal in populations at equilibrium because it represents the stochastic shape of gene trees, although it is generally unimodal in situations of recent demographic expansion (Slatkin & Hudson, 1991; Rogers & Harpending, 1992) or range expansions with high levels of migration between neighbouring demes (Ray, Currat & Excoffier, 2003; Excoffier, 2004). The frequency spectrum of mutations was examined using Tajima's  $D$  (Tajima, 1989) and Fu's  $F_s$  (Fu, 1997).

The time-frame of colonization of Mallorca by rabbits and hares was estimated using: (1) the time of the most recent common ancestor (TMRCA) of the Mallorcan clades and (2) the TMRCA of the clades conveying Mallorcan and the most closely related continental haplotypes. Estimates of the TMRCA were performed using BEAST, version 1.7.4 (Drummond *et al.*, 2012) on three separate datasets corresponding to the major lineages detected (one for rabbits and two for hares). The best-fitted mutation model was determined by JMODELTEST, version 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008) using the corrected Akaike information criterion. This best model or, if not implemented in BEAST, the next-most complex model available and a strict molecular clock were assigned to the data. The demographic model selected was the Bayesian skyline plot, which imposes fewer demographic prior assumptions than the remainder of the available models (Drummond *et al.*, 2005). Several runs with different chain lengths were performed to assess the consistency of the estimates across independent runs. The final analyses were run three times with a number of iterations that varied between 50 and 150 million depending on the run length needed to obtain stability of the Markov chain and high effective sample sizes of the parameter estimates. TRACER, version 1.5 (Rambaut & Drummond, 2007) was used to assess convergence of the runs. The first 10% of the samples of the Markov chain of each run were discarded as burn-in and the tree files of the independent runs were combined using LOGCOMBINER, version 1.7.4, included in the BEAST package. The resulting trees were then visualized in FIGTREE, version 1.3.1. Melo-Ferreira *et al.* (2007) estimated a divergence rate of  $0.158 \text{ Myr}^{-1}$

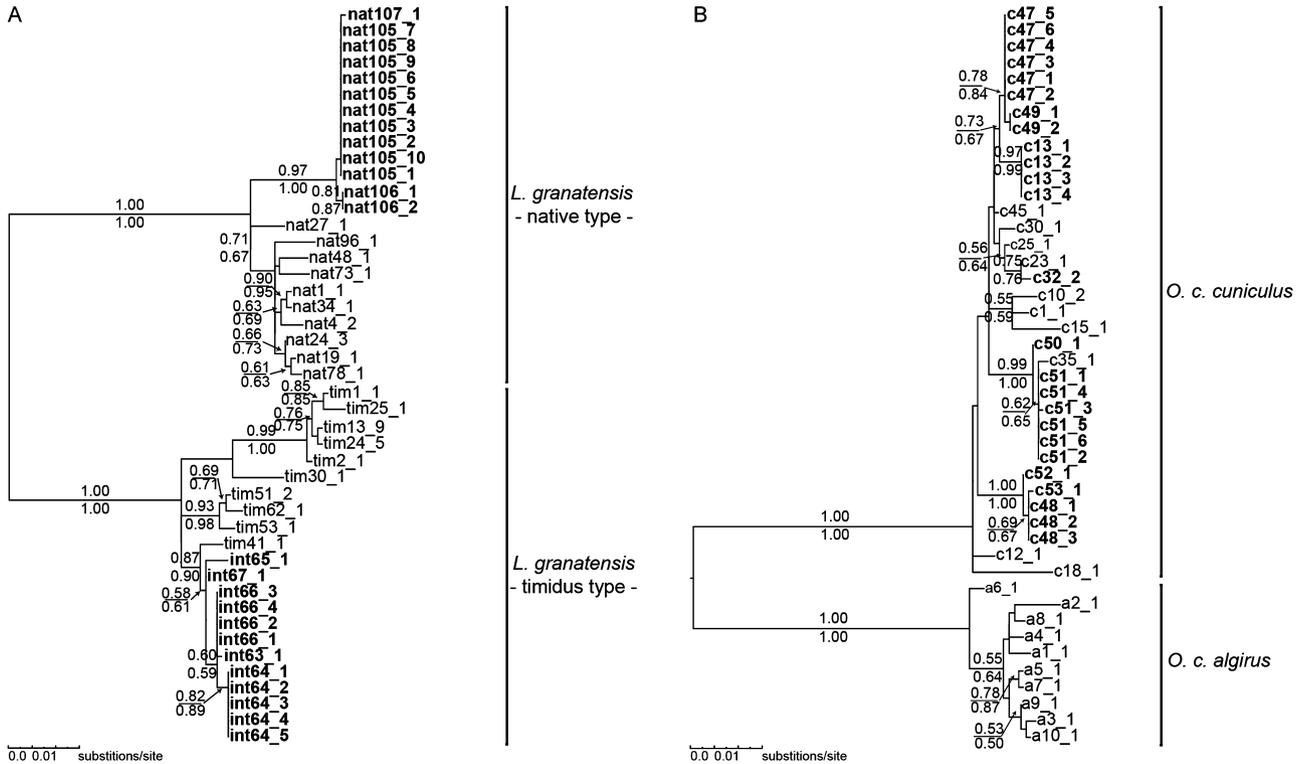
for the concatenated fragments of cytochrome *b* and D-loop, from which a divergence rate of  $0.312 \text{ Myr}^{-1}$  for the D-loop can be derived. This rate was also used for the rabbit analysis.

The demographic history of the Mallorcan lineages was further explored using the Bayesian skyline plot and four additional demographic models were implemented in BEAST, version 1.7.4: constant population size, exponential growth, logistic growth, and expansion growth. The fit of the different models was compared by calculating the Bayes factor (BF) (Newton & Raftery, 1994) from the ratio of the marginal likelihoods of the different models given by the harmonic mean estimator (Suchard, Weiss & Sinsheimer, 2001) as implemented in TRACER, version 1.5. Because the harmonic mean estimator may perform poorly, in addition, the path sampling method was used to calculate marginal likelihoods, and the BF's of the different models were again estimated (Baele *et al.*, 2012).

## RESULTS

### GENETIC DIVERSITY AND RELATIONSHIP BETWEEN CONTINENTAL AND MALLORCAN HAPLOTYPES

All analyzed Mallorcan hare samples were assigned as *L. granatensis* according to the diagnostic nuclear marker (UCP2 locus); a 23-bp deletion was present in both alleles of this locus in all analyzed individuals (Melo-Ferreira *et al.*, 2009). The phylogenetic reconstruction based on control region sequences (450 bp) showed that 52% of analyzed *L. granatensis* from Mallorca had native mtDNA haplotypes and 48% possessed the *timidus*-like mtDNA, similar to the observed frequency in northern Iberia (Melo-Ferreira *et al.*, 2005) (Fig. 2A). A total of eight different haplotypes were identified among the 25 hare samples from Mallorca (five of *granatensis* type and three of *timidus* type), and a high nucleotide diversity was found ( $\pi_{\%} = 6.56$ ; Table 1), which reflected the divergence between native and introgressed haplotypes. Combining these sequences and those from various Iberian populations (Fig. 3; see also Supporting information, Fig. S1), the continental haplotypes that most resembled haplotypes of the Mallorcan native *L. granatensis* were those from the population of Caceres (Fig. 3A; see also Supporting information, Fig. S1a). For *L. granatensis* with *timidus*-like mtDNA, the closest continental haplotype to the Mallorcan haplotypes was detected in Zaragoza (Fig. 3B). Analyzing the two evolutionary lineages separately, diversity among Mallorcan haplotypes (native:  $h = 0.410$ ,  $\pi_{\%} = 0.16$ ,  $\theta_{S\%} = 0.21$ ; *timidus*-like:  $h = 0.758$ ,  $\pi_{\%} = 0.63$ ,  $\theta_{S\%} = 0.67$ ) was generally lower than that observed in the continent ( $h = 0.987$ ,  $\pi_{\%} = 2.44$ ,  $\theta_{S\%} = 3.62$ ;  $h = 0.972$ ,  $\pi_{\%} = 3.83$ ,  $\theta_{S\%} = 3.02$ ;

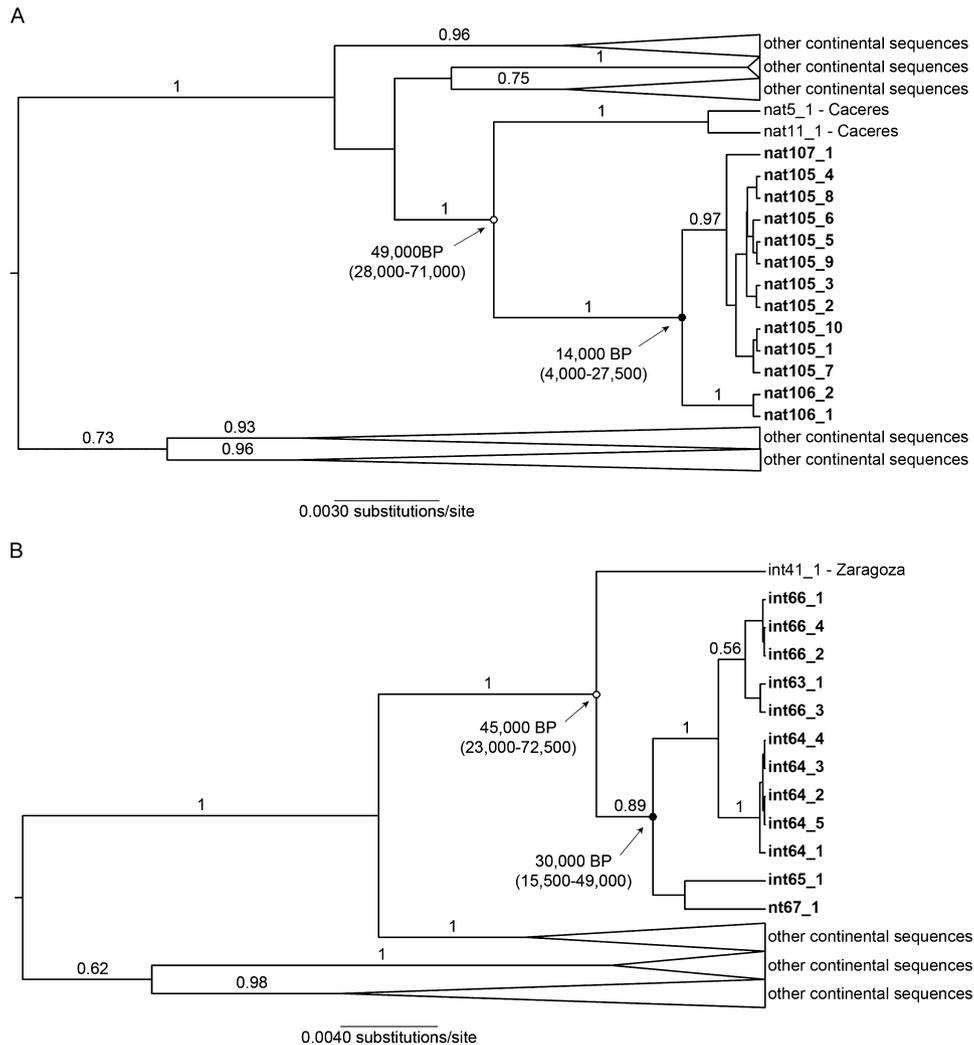


**Figure 2.** Maximum likelihood (ML) phylogenies showing relationships between sampled hares (A) and rabbits (B) and the known lineages that exist in each species (a subset of available continental sequences was used). ML and Neighbour-joining bootstrap values are depicted above and below branches, respectively. Mallorcan individuals are shown in bold (detailed information on each haplotype is provided in the Supporting information, Table S1).

**Table 1.** Estimates of sequence diversity and neutrality tests in native (nat) and introgressed (*tim*-like) hares (*Lepus granatensis*) and rabbits (*Oryctolagus cuniculus cuniculus*)

Group	<i>N</i>	<i>N<sub>H</sub></i>	<i>h</i>	$\pi$ (%)	$\theta_s$ (%)	Tajima's <i>D</i>	Fu's <i>F<sub>s</sub></i>
<b>Hares</b>							
<i>gra</i> , IP	429	166	0.990 (0.001)	7.26 (3.51)	4.56 (0.94)	1.37	-23.43
<i>gra</i> , Maj	25	8	0.793 (0.061)	6.56 (3.31)	3.19 (1.09)	3.24	14.39
nat, IP	234	104	0.987 (0.002)	2.44 (1.23)	3.62 (0.83)	-1.05	-24.03*
nat, Cac	10	8	0.933 (0.077)	2.79 (1.56)	2.58 (1.11)	-0.81	0.12
nat, Maj	13	3	0.410 (0.154)	0.16 (0.14)	0.21 (0.14)	-0.1	0.18
<i>tim</i> -like, IP	195	62	0.972 (0.004)	3.83 (1.89)	3.02 (0.73)	0.8	-7.74
<i>tim</i> -like, IP (lin.B)	95	37	0.955 (0.009)	2.15 (1.10)	2.49 (0.68)	-0.45	-7.73
<i>tim</i> -like, Zar (lin.B)	18	7	0.634 (0.127)	0.78 (0.47)	1.37 (0.55)	-1.69*	0.19
<i>tim</i> -like, Maj	12	5	0.758 (0.093)	0.63 (0.40)	0.67 (0.33)	-0.65	0.63
<b>Rabbits</b>							
IP	32	21	0.972 (0.014)	2.61 (1.35)	2.98 (0.98)	-0.77	-3.54
Fra	28	26	0.995 (0.011)	2.06 (1.09)	2.61 (0.89)	-0.89	-16.93
Maj	25	9	0.867 (0.037)	2.40 (1.26)	1.77 (0.64)	0.87	3.83

*gra*, *Lepus granatensis*; nat, *Lepus granatensis* with native mitochondrial DNA haplotypes; *tim*-like, *Lepus granatensis* with *timidus*-like haplotypes; IP, Iberian Peninsula; Fra, France; Maj, Mallorca; Cac, Caceres; Zar, Zaragoza; *N*, number of individuals analyzed; *N<sub>H</sub>*, number of observed mtDNA haplotypes; *h*, haplotype diversity;  $\pi$ , nucleotide diversity;  $\theta_s$ , computed from the number of segregating sites. SDs are shown in brackets. Significant values are indicated by an asterisk.

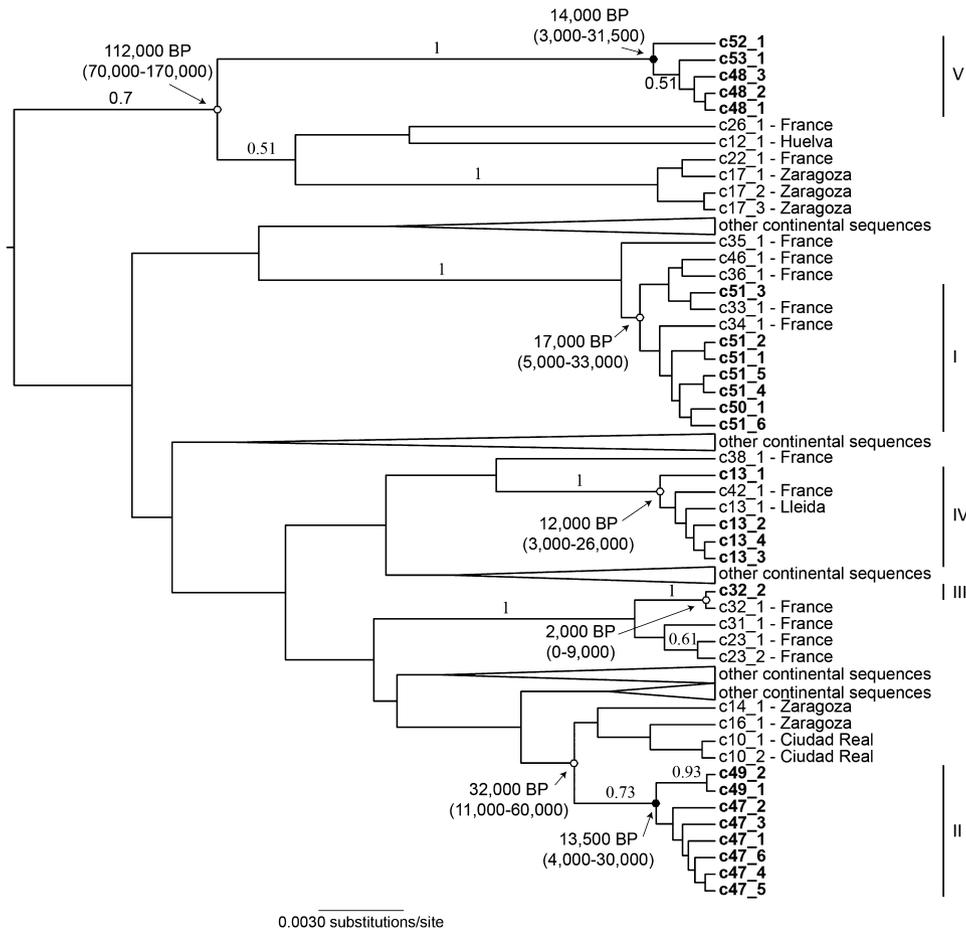


**Figure 3.** Bayesian inference (BI) phylogenetic tree reconstructed in BEAST for the complete dataset (continental and Mallorcan sequences) of the two major hare mitochondrial DNA lineages detected in Mallorca: native *Lepus granatensis* (A) and *timidus*-like *L. granatensis* (B). Posterior probabilities higher than 0.5 are shown above branches. Continental clades are collapsed except those closely related to the haplotypes found in Mallorca (locality is indicated). Nodes for which the times of the most recent common ancestor were inferred are depicted by hexagons: black, Mallorcan clades; white, clades grouping Mallorcan and the most closely-related continental sequences.

for native and *timidus*-like *L. granatensis*, respectively) (Table 1).

The ML and NJ phylogenetic reconstruction suggested that all Mallorcan rabbits belonged to mtDNA clade B, which has been attributed to the subspecies *O. c. cuniculus*, native to north-eastern Iberia and France (Branco *et al.*, 2002) (Fig. 2B). Therefore, in the subsequent analyses, a dataset with only clade B individuals from the continental range of the species was added. Nine haplotypes were found among the analyzed Mallorcan rabbits and levels of sequence diversity were comparable to those found in the native range of the subspecies *O. c. cuniculus* (Table 1). When comparing the haplotypes found in

Mallorca with those from the Iberian Peninsula and French populations (Fig. 4; see also Supporting information, Fig. S2), the Mallorcan rabbits do not form a monophyletic group. Two similar haplotypes (hereafter named I) were more closely related to French haplotypes, and two individuals (II) were more closely related to a haplotype that included individuals from Zaragoza, Benavente, and France. Mallorcan individuals shared the same haplotype with specimens from the continent twice. In one case, a haplotype (III) harboured one Mallorcan individual and one French individual. In the other case, a haplotype (IV) included four Mallorcan individuals and one individual from Lleida. Finally, one last haplogroup (V)



**Figure 4.** Bayesian inference (BI) phylogenetic tree reconstructed in BEAST for the complete dataset (continental and Mallorcan sequences) of the rabbit mitochondrial DNA lineage detected in Mallorca: *Oryctolagus cuniculus cuniculus*. Posterior probability higher than 0.5 are shown above branches. Continental clades are collapsed except those closely related to the haplotypes found in Mallorca (locality is indicated). Nodes for which the times of the most recent common ancestor were calculated are depicted by hexagons: black, Mallorcan clades; white, clades grouping Mallorcan and the most closely-related continental haplotypes. Roman numbers depict the five identified Mallorcan haplogroups.

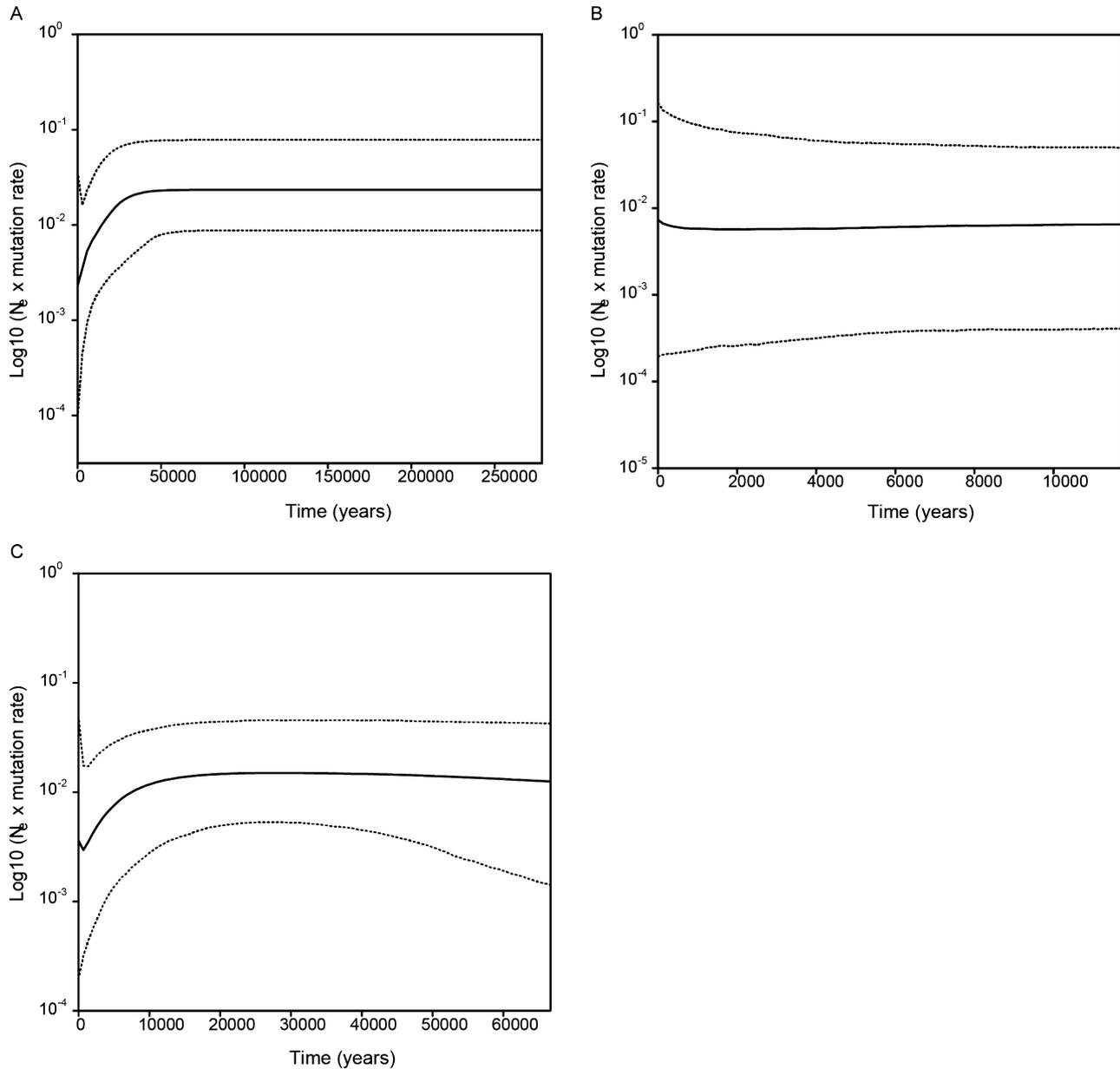
was highly divergent from the other sampled rabbit haplotypes.

#### DEMOGRAPHIC ANALYSIS AND TIME OF THE MOST RECENT COMMON ANCESTOR

The mismatch analysis of both Mallorcan native and *timidus*-like *L. granatensis* did not reject the sudden expansion model (see Supporting information, Fig. S3). However, although Tajima's *D* value was negative in these two groups (Table 1), both values were not significant ( $P > 0.05$ ). On the other hand, Fu's  $F_s$  values were positive, both for native and *timidus*-like hares but, again, neither was significant ( $P > 0.02$ ) (Table 1). The Bayesian skyline analysis of *timidus*-like *L. granatensis* did not suggest recent expansion (Fig. 5) and, in the case of native *L. granatensis*, the Markov chain Monte Carlo failed

to converge after multiple runs (e.g. 200 million generations), likely as a result of the low levels of variation present in the alignment (this plot is not shown). The BF comparison of the different demographic models did not strongly favour any of the demographic expansion models over the constant population size model for both the Mallorcan *timidus*-like and native *L. granatensis* datasets [largest  $2\ln(\text{BF})$  favouring a growth model of approximately 2.5; for a description of BFs, see Kass & Raftery, 1995] (see Supporting information, Tables S2, S3).

The mismatch analysis of the sequences of Mallorcan rabbit specimens showed a multimodal distribution, rejecting the sudden expansion model (see Supporting information, Fig. S3). Tajima's *D* and Fu's  $F_s$  values were both positive, although neither was significant ( $P > 0.05$  and  $P > 0.02$ , respectively) (Table 1). The Bayesian skyline analysis did not



**Figure 5.** Bayesian skyline plots of *Lepus granatensis* Mallorcan haplotypes (including both *L. granatensis* native and *timidus*-like haplotypes) (A), *L. granatensis* Mallorcan *timidus*-like haplotypes (B), and *Oryctolagus cuniculus cuniculus* Mallorcan haplotypes (C). Solid lines represent the median effective population size and dashed lines the 95% highest posterior density (HPD) upper and lower bounds. On the y-axis, the effective population sizes ( $N_e$ ) are scaled to the mutation rate, whereas the x-axis represents coalescent time in years. The plot of Mallorcan native *L. granatensis* haplotypes is not shown given the lack of convergence among runs.

suggest recent population expansion (Fig. 5) and, again, no strong support was obtained for the expansion models relative to the constant population size model [largest  $2\ln(\text{BF})$  favouring a growth model of approximately 2.2; for a description of BFs, see Kass & Raftery, 1995] (see Supporting information, Tables S2, S3).

Estimates of the dates of introduction of hares and rabbits in Mallorca were derived from the TMRCA both for the Mallorcan clades and for the closest continental haplotype (Figs 3, 4). For native *L. granatensis* haplotypes, the highest posterior density (HPD) 95% confidence interval (CI) of the TMRCA of the Mallorcan variants is from 27 500 years

BP to 4000 years BP, whereas the TMRCA with the closest continental haplotype is between 71 000 BP and 28 000 BP (Fig. 3A). In the case of *timidus*-like *L. granatensis*, the HPD 95% values of the Mallorcan TMRCA range from 49 000 BP to 15 500 BP, whereas that including the continental haplotypes ranges from 72 500 to 23 000 years BP (Fig. 3B).

The trees obtained from the analysis using BEAST suggest that at least five independent rabbit introductions may have occurred (Fig. 4), in accordance with the network analyses (see Supporting information, Fig. S3). Whenever the Mallorcan haplotypes did not form a monophyletic group (haplogroups I and IV) or the colonization event was represented by a single individual (haplogroup III), the TMRCA was only calculated including the more closely-related continental specimen. Overall, the 95% HPD intervals varied between 170 000 years BP (group V) and the present (groups III). The detailed time estimates for the colonization of all rabbit haplogroups and the 95% HPD are shown in Figure 4.

## DISCUSSION

### THE NATURE OF MALLORCAN HARES AND RABBITS

In accordance with previous descriptions of Mallorcan hares (Palacios & Fernández, 1992), our results on the analyzed nuclear gene suggest that the hares included in the present study are all Iberian hares, *L. granatensis*. In addition, based on mtDNA analyses, the genetic variation detected among Mallorcan hares is well within and not divergent from the continental populations and therefore no support was found in our data for their classification as a separate subspecies, *L. g. solisi*, as suggested by Palacios & Fernández (1992). However, because subspecific classification must rely on information from multiple sources (including morphological data), a more thorough assessment is needed to address this question.

All of the Mallorcan rabbits analyzed in the present study belong to the subspecies *O. c. cuniculus*, for which the original distribution is north-eastern Iberia and France. This contrasts with previous suggestions for the introduction of the *O. c. algirus* subspecies in Mallorca by the Phoenicians, which is endemic to south-western Iberia (Gibb, 1990). Although it is not known whether the latter subspecies was present on the island but went extinct, our results suggest that *O. c. algirus* may be currently absent from the island, even though a more thorough sampling would be needed to confirm this hypothesis. In the present study, we found no evidence of the existence of the mythological 'esquirol'. Such an assumption may have been fueled by the fact that *O. c. cuniculus* is the largest of the subspecies of the European rabbit

(Ferreira, 2011). Eventual sporadic island gigantism (which is unknown for Mallorcan rabbits) or the presence of an extinct giant rabbit in the Balearics (classified as *Nuralagus rex*) in the fossil record (Quintana, Köhler & Moyà-Solà, 2011) may have also contributed to the myth.

### ORIGIN AND TIME-FRAME OF THE MULTIPLE COLONIZATIONS OF MALLORCA BY LAGOMORPHS

The last time that Mallorca was in contact with the Iberian Peninsula, allowing for natural colonization of land mammals, was possibly during the Messinian crisis, some 5–5.5 Myr BP (Hsu *et al.*, 1977; Azzaroli & Guazzone, 1980). Such natural colonization of lagomorphs is unlikely because previous studies suggest that rabbits and hares have been introduced after the first human arrival at Mallorca, which is considered to have occurred by the fifth millennium BP (4300–4100 BP) (Alcover, 2008).

The haplotype network and phylogenetic analyses, combining sequences from Mallorca and continental Europe, show that neither Mallorcan hares, nor rabbits are monophyletic, suggesting that multiple colonizations occurred (Figs 3, 4; see also Supporting information, Figs S1, S2). A minimum of two colonizations may have occurred for hares and five for rabbits. Using the mtDNA TMRCA estimates between the Mallorcan and continental haplotypes, we estimated the likely time-frame of the colonization events, and, in case of a recent colonization, also determined the likely continental origin of such colonizers, bearing in mind that Quaternary glaciations induced major changes in the distribution of species (Hewitt, 2000), including rabbits and hares (Branco *et al.*, 2002; Melo-Ferreira *et al.*, 2007). Hares were estimated to have colonized the island between 4000 and 72 500 BP (HPD 95% CI), whereas overall rabbit introductions were suggested to have occurred between 170 000 BP and the present (Figs 3, 4). Two strategies were adopted as a proxy of possible colonization times: (1) the TMRCA of Mallorcan clades and (2) the TMRCA of Mallorcan and closest continental relative. Although (2) may tend to overestimate colonization time if we failed to sample the closest continental relative, (1) may underestimate colonization time if colonizer haplotypes went extinct, or overestimate it if multiple haplotypes from the clade were originally introduced and the extant haplotypes coalesce in a continental ancestral. Our estimates must therefore be interpreted with caution because of the various uncertainties that derive from the use of molecular calibrations to estimate evolutionary events (Graur & Martin, 2004; Heads, 2005). This is reflected in the wide intervals of possible colonization times obtained, which may also suggest a more

complex colonization history. However, these inferences provide important clues about the time-frame of colonization on a broader scale. The known first human arrival in Mallorca (fifth millennium BP) falls generally within the intervals of the estimates of TMRCA of Mallorcan clades and, even when considering the TMRCA with the closest continental relative (which is possibly largely overestimated), these intervals are dramatically more recent than the last land bridge that would have allowed natural colonization 5 Mya. Our results suggest that the extant populations of hares and rabbits in Mallorca are recent and most likely result from recent human-mediated introductions.

Considering the evolutionary proximity between Mallorcan and continental haplotypes, hares appear to have been introduced from two very restricted regions in the Iberian Peninsula: Cáceres and Zaragoza, possibly around the same time scale (95% HPD of the TMRCA estimates overlap). Mallorcan rabbits appear to have been introduced into Mallorca at least in five independent events possibly originating from distinct regions from Iberia and France (the regions of Benavente, Zaragoza and eventually Lleida, as well as France possess the most closely-related haplotypes) (Fig. 4). Interestingly, the divergence of the Mallorca rabbits from the continental haplotypes is quite variable, suggesting a more recurrent dynamic of introductions over a longer period of time. This contrasts with the patterns inferred from hares, which suggest a more simple and restricted regime of introduction.

The introduction of lagomorphs in Mallorca thus appears to be intimately related to human movements in the Mediterranean and settlements in Mallorca in historical and more recent times. A first human settlement in Mallorca likely occurred approximately 4300–4100 years BP (Alcover, 2008), followed by the emergence of the Talayotic culture (end of the fourth and beginning of the third millennium BP), and then by the occupation of the island by the Romans (2073 years BP). The first settlers are assumed to have introduced into Mallorca four domestic species (sheep, goats, cows, and pigs) and two wild rodents: the woodmouse (*Apodemus sylvaticus* Linnaeus, 1758) and the garden dormouse (*Eliomys quercinus* Linnaeus 1766) (Alcover, 2008; Bover & Alcover, 2008). Both rodent species have been shown to have a probable Iberian or French origin (Ramalhinho & Libois, 2001; Michaux *et al.*, 1998; Perez, Libois & Nieberding, 2013). It is difficult to assess whether the coincidence of geographical origins of these species and hares (Iberia) and rabbits (Iberia and France) inferred reflects a coincidence of introduction events. Also, it is uncertain whether rabbits were introduced during this settlement or

only later by the Romans. During the Roman period, several mammals may have been introduced, such as rabbits, house mouse (*Mus musculus* Linnaeus, 1758), and weasels (*Mustela nivalis* Linnaeus, 1766) (Bover & Alcover, 2008). These two civilizations may have contributed to several waves of introductions. For example, the Talayotic people are known to have acted as mercenaries in the Punic Wars, which were fought in Sardinia, Sicily, mainland Italy, the Iberian Peninsula, and North Africa (2214–2096 years BP), whereas the expansion of the Roman empire established frequent connections to the European mainland. Thus, either by commerce or by the return of mercenaries to the island, there could be importation of animals from the continent, which may have not only more frequently included rabbits, but also hares, according to our results. However, we cannot discard the possibility that both lagomorph species were introduced earlier, during undocumented human movements in the Mediterranean (some of our TMRCA estimates are rather old; Figs 3, 4) or, in some cases, more recently for hunting purposes (e.g. the lower bound of the 95% HPD of the TMRCA of rabbit haplogroup III and the continental haplotype is zero; Fig. 4).

#### DEMOGRAPHY OF MALLORCAN LAGOMORPH POPULATIONS

The colonization of islands usually results in a strong bottleneck of genetic diversity (as a result of the founder event) followed by rapid expansion because the colonizers encounter an empty niche to inhabit and adapt to. We tested this hypothesis for Mallorcan rabbits and hares.

The genetic diversity of hares, after distinguishing the two lineages present in Mallorca (native and *timidus*-like), appears to have decreased in both lineages ( $h = 0.410$ ,  $\pi_{\%} = 0.16$ ,  $\theta_{S\%} = 0.21$ ;  $h = 0.758$ ,  $\pi_{\%} = 0.63$ ,  $\theta_{S\%} = 0.67$ ; native and *timidus*-like *L. granatensis*, respectively) compared to the diversity in continental ones ( $h = 0.987$ ,  $\pi_{\%} = 2.44$ ,  $\theta_{S\%} = 3.62$ ;  $h = 0.972$ ,  $\pi_{\%} = 3.83$ ,  $\theta_{S\%} = 3.02$ ; for native and *timidus*-like *L. granatensis*, respectively) (Table 1), as expected during founder events and colonization (Nei, Maruyama & Chakraborty, 1975). We have further estimated the levels of diversity for the more closely-related continental populations, using all of the sequences available in GenBank (10 native and 18 *timidus*-like *L. granatensis* from Cáceres and Zaragoza, respectively). Again, both present higher genetic diversity than the two lineages present in Mallorca (Table 1), suggesting a decrease in diversity as a result of the founder events. After colonization, and according to BF comparison, the demographic model of constant population size cannot be ruled

out (see Supporting information, Tables S2, S3). Also, although, for both lineages of hares in Mallorca, the mismatch distribution did not reject the Sudden Expansion Model (see Supporting information, Fig. S3), no significant deviation to mutation drift expectations was found by Tajima's  $D$  and Fu's  $F_s$  (Table 1). For rabbits, no signs of demographic expansion were found: the mismatch distribution rejected the Sudden Expansion Model (see Supporting information, Fig. S3); Tajima's  $D$  and Fu's  $F_s$  values were both positive and not significant (Table 1); and the Bayesian skyline analysis did not suggest any recent population expansion (Fig. 5). In addition, demographic expansion models were not favoured, as attested by the BF comparison (see Supporting information, Tables S2, S3). Although a single marker is being analyzed, the lack of evidence for population expansion may also result from the fact that introductions were very recent and the genetic signals of a bottleneck are still present in the genetic diversity of Mallorcan lagomorphs. However, the small number of analyzed specimens in the present study may have been insufficient to properly infer the demography of the Mallorcan populations and explain our results. Therefore, further work is needed to confirm the demographic history of both founder and post-introduction events for Mallorcan hare and rabbit populations.

#### CONCLUSIONS

The present study shows that rabbits and hares from Mallorca originated from the complex dynamics of translocations of fauna induced by the rich history of human settlements and colonizations in the Mediterranean basin. By contrast to previous expectations, the European rabbit subspecies that inhabits the island is *O. c. cuniculus*, and we confirm that Mallorcan hares belong to the species *L. granatensis*. Given the complex dynamics of introductions found particularly for the European rabbit, this may reflect recurrent waves of faunal replacements in Mallorca induced from repeated translocations. Human-mediated introductions have shaped the geographical distribution of Mediterranean fauna and future work may help our understanding of the detailed history and evolutionary consequences of this complex and heterogeneous process.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1.** Median-joining network of *Lepus granatensis* haplotypes found in Mallorca (black; haplotype sizes proportional to frequencies) and in the Iberian Peninsula (white) (a, native haplotypes; b, *timidus*-like haplotypes). Mallorcan haplotype names are indicated followed by their frequency in brackets. Branches are generally proportional to the number of differences between haplotypes, and black dots on branches indicate mutational steps. Dashed circles around haplotypes indicate the closest continental haplotypes.

**Figure S2.** Median-joining network of the rabbit lineage B haplotypes found in Mallorca (black; haplotype sizes proportional to frequencies), Iberian Peninsula (white), and France (grey). Mallorcan haplotype names are indicated followed by their frequency in brackets. Branches are generally proportional to the number of differences between haplotypes, and black dots on branches indicate mutational steps. Dashed circles around haplotypes indicate the Mallorcan haplogroups.

**Figure S3.** Observed (bars) and expected under a sudden expansion model (solid lines) mismatch distributions of (a) Mallorcan native *Lepus granatensis*; (b) Mallorcan *timidus*-like *L. granatensis*; (c) Mallorcan *Oryctolagus cuniculus cuniculus*. The number of pairwise differences is represented on the x-axis.

**Table S1.** Populations of hares and rabbits sampled and respective mitochondrial DNA haplotypes (the absolute frequency is shown in parenthesis).

**Table S2.** Bayes factor (2lnBF) estimated from the marginal likelihood calculated with the harmonic mean estimator of different demographic models applied to the Mallorcan hares and rabbits.

**Table S3.** Bayes factor (2lnBF) estimated from the marginal likelihood calculated with the path sampling procedure of different demographic models applied to the Mallorcan hares and rabbits.