1　**Postglacial range revolutions in South European hares (*Lepus* spp.): insights from**
2　**ancient DNA and ecological niche modelling**
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5　Running title: Range revolutions in South European hares
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46   **Abstract**
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48   **Aim**. The distribution of hares (*Lepus* spp.) in southern Europe was markedly different at the
49   last glacial maximum. Historical hybridization during range replacements led to high
50   frequencies of mtDNA introgression from *Lepus timidus* into populations of three other
51   species in northern Iberian Peninsula, even though *L. timidus* subsequently disappeared from
52   the region. We use paleontological records to provide new insights on the biogeographic
53   history of these range replacements, combining ancient DNA and ecological niche modelling.
54   **Location**. Southern France.
55   **Taxon**. Hares (genus *Lepus*).
56   **Methods**. DNA was extracted from hare bones collected at Neolithic sites in Southern France
57   (5.5-7.5 thousand years before present; kyr BP) with uncertain species assignment. One
58   mtDNA fragment was sequenced and 27 SNPs from 11 nuclear genes with species-diagnostic
59   information were genotyped. Distributions of *L. granatensis*, *L. europaeus* and *L. timidus*
60   were modelled using spatial and bioclimatic predictors, and the favourability function. Each
61   model was transferred to 6 kyr BP and favourabilities were combined to determine the species
62   with the highest environmental favourability in each sampled locality.
63   **Results**. Ancient DNA analysis showed that the screened specimens belong to *L. granatensis*,
64   a species presently confined to the Iberian Peninsula, but carried mtDNA haplotypes from *L.
65   timidus*. Niche models show that *L. granatensis* achieved highest favourability in Southern
66   France 6 kyr BP reinforcing the molecular evidences.
67   **Main conclusions**. *L. granatensis* replaced *L. timidus* populations from Iberia to Southern
68   France, being present in this region 5.5 kyr BP. Subsequent westwards invasion of *L.
69   europaeus* likely replaced these *L. granatensis* populations towards northern Iberia,
70   establishing current ranges. Despite successive species replacements, introgressed mtDNA
71   haplotypes from *L. timidus* mark the distribution of the species in northern Iberian Peninsula
72   before it disappeared in the region. Characterizing complex biogeographic histories of
73   interacting species is key to understand processes that led to current distributions of genetic
74   diversity.
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77   **Keywords**: Range replacements; Ancient DNA; Niche models; Hybridization; Introgression;
78   Hares.

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1. Introduction

The geographic distribution of species is a dynamic expression of abiotic and biotic factors that determine their ability to persist in a given place (Sexton *et al.*, 2009; Wiens, 2011). Climate change, as that driven by the glacial cycles, induced major changes in environmental conditions and consequently led to important range changes of species according to niche conservatism, as well as their adaptations and ability to disperse (Stewart *et al.*, 2010). Species adapted to temperate climates tend to have contracted their ranges during glacial periods and expanded during interglacials (Hewitt, 2000), but reverse patterns have been inferred for arctic species adapted to colder climates (Flagstad & Røed, 2003; Dalén *et al.*, 2005; Stewart & Dalén, 2008). However, the distribution of species is not only influenced by abiotic conditions, but also by interactions with other species that occupy overlapping ecological niches (Sexton *et al.*, 2009; Godsoe *et al.*, 2017). This is particularly relevant for parapatric pairs of closely related species that share ecological niche properties and tend to competitively exclude each other (Acevedo *et al.*, 2012a; Reino *et al.*, 2017). In addition, hybridization during range replacements may lead to introgression into the invading species in the newly colonized territories. This can be driven by the demographic process and repeated hybridization (Currat *et al.*, 2008; Excoffier *et al.*, 2009), or facilitate the invasion through the incorporation of advantageous variants (Rieseberg *et al.*, 2007; Hedrick, 2013).

Paleontological, genetic and ecological data suggest that hares (*Lepus* spp.) in southern Europe markedly changed their ranges since the last glacial maximum (LGM). The mountain hare, *Lepus timidus*, is currently widely distributed in the northern Palearctic and in some isolated populations in Ireland, Scotland, Poland and the Alps (Angerbjörn & Flux, 1995), but Pleistocene paleontological records show it was present further south in the past, such as in southern France and northern Iberian Peninsula (Altuna, 1970; Lopez-Martinez, 1980). The current range of the species thus likely represents colonization of deglaciated areas from glacial populations (Smith *et al.*, 2017), in agreement with expectations of range changes through glacial cycles for cold-adapted species (Stewart *et al.*, 2010). An ancient DNA study suggested that *L. timidus* maintained a continuous population south of the ice margin during the last glacial period and was present in Western and Southern Europe after the LGM (Smith *et al.*, 2017). In keeping, ecological niche models show the presence of areas favourable for *L. timidus* in the southern European Peninsulas at the LGM (Acevedo *et al.*, 2015). The presence of *L. timidus* in the Iberian Peninsula also left traces on the genetic variation of extant species through historical introgressive hybridization (Melo-Ferreira *et al.*, 2005; Alves *et al.*, 2008b; Melo-Ferreira *et al.*, 2009; Melo-Ferreira *et al.*, 2012; Melo-Ferreira *et al.*, 2014). High frequencies of mtDNA of *L. timidus* origin were found in the northern populations of the Iberian hare, *L. granatensis*, an Iberian endemic that occupies most of the peninsula, and the European hare, *L. europaeus*, which inhabits the northernmost part and extends its range towards Central and Eastern Europe (Melo-Ferreira *et al.*, 2005). In the other Iberian species, the broom hare, *L. castroviejoi*, present in the Cantabrian Mountain chain, the native mtDNA has been completely replaced by that of *L. timidus* presumably during the Pleistocene (Melo-Ferreira *et al.*, 2012). These historical hybridization events also affected the nuclear genome, as introgression of nuclear DNA was generally found at low frequencies but widespread, though more frequent in northern Iberia (Melo-Ferreira *et al.*, 2009; Seixas *et al.*, 2018). Patterns of ancient introgression into *L. granatensis* are compatible with a northwards postglacial expansion of the species from southwest Iberia (Marques *et al.*, 2017) and replacement of historical local populations of *L. timidus* in the North with repeated hybridization in the invasion front (Melo-Ferreira *et al.*, 2007; Melo-Ferreira *et al.*, 2009; Melo-Ferreira *et al.*, 2011; Seixas *et al.*, 2018). During the postglacial westwards invasion of

146   *L. europaeus* (Lopez-Martinez, 1980; Pierpaoli *et al.*, 2003; Stamatis *et al.*, 2009; Forest &
147   Cheylan, 2015), the species could have then replaced *L. timidus* and/or *L. granatensis*
148   populations, though indirect inferences from mtDNA introgression patterns favour a direct
149   contact with *L. granatensis* in the invasion of northern Iberia (see Melo-Ferreira *et al.*, 2014).
150
151   Here, we analyse hare bone remains collected in Neolithic sites (5.5-7.5 thousand years before
152   present; kyr BP) in Southern France, i.e. possibly pre-dating the westwards invasion of *L.*
153   *europaeus*, to provide new insights onto the past biogeography of hare species in southern
154   Europe. The bones were attributed to an unknown hare species coined "petit lièvre des
155   Vautes" (little hare from Les Vautes) (Forest, 2003; Forest & Cheylan, 2015; Escallon &
156   Fritz, 2016). They show *Lepus* morphological features that separate them from the European
157   rabbit (*Oryctolagus cuniculus*) but are smaller than *L. europaeus* (Callou, 1997; Forest &
158   Cheylan, 2015). The small size of the bones suggest they may belong to extant Iberian or
159   Italian species, which would therefore have been replaced in southern France by *L. europaeus*
160   more recently (Forest & Cheylan, 2015). We identify the species of provenance using both
161   mitochondrial and nuclear ancient DNA and perform ecological niche modelling to clarify the
162   extent and sequence of range changes of hares in southern Europe.
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165   **2. Material and Methods**
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167   *2.1. Samples and DNA extraction*
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169   Bone samples, collected at Neolithic archaeological sites in Languedoc-Roussillon, France,
170   were used in this work (Table 1) (Forest & Cheylan, 2015; Escallon & Fritz, 2016). Samples
171   were taken in the ancient DNA laboratories of the Swedish Museum of Natural History using
172   a Dremel drill to obtain approximately 50 mg of bone powder from each specimen. DNA was
173   extracted using a modified version of the silica-based ancient DNA extraction protocol from
174   Yang *et al.* (1998) as reported in Ersmark *et al.* (2015). Standard precautions for ancient DNA
175   work were taken, including the use of negative controls in all extractions and sterilization of
176   equipment plastic wares and reagents using bleach or UV light. In brief, the bone powder was
177   incubated with EDTA buffer, urea and proteinase K, under permanent motion for 24 hours at
178   55 °C. In an Aura PCR cabinet, the supernatant was transferred to Vivaspin filters, mixed
179   with PB buffer and transferred to Qiaquick spin columns. The columns were then washed
180   using PE buffer, and DNA was eluted with 50 µL of EB buffer.
181
182
183   Table 1: Sampling information.

Code	Years BP	Municipality	Site	Collection Year	Bone
SL002	7000	Mauguio	Péras 1	2013	Femur, diaphysis
SL005	5500	Saint-Gély-du-Fesc	Les Vautes	1996	Femur, diaphysis
SL007	5500	Saint-Gély-du-Fesc	Les Vautes	1996	Tibia, diaphysis
SL012	5500	Saint-Gély-du-Fesc	Les Vautes	1996	Metatarsus 2, whole, right
SL014	5500	Paulhan	Puech-Haut	1999	Metatarsus 2, whole, left

184   BP = Before present.
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189   *2.2. DNA sequencing and genotyping*

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191   A fragment of mitochondrial Cytochrome *b* was amplified from the ancient DNA extracts
192   (including the negative controls), using five short overlapping PCRs (from Smith *et al.*, 2017;
193   PCR information in Table S1 in Appendix S1). Amplification success was tested in standard
194   agarose gel electrophoreses. Amplicons were purified using Exonuclease I and Shrimp
195   Alkaline Phosphatase (USB® ExoSAP-IT® PCR Product Cleanup, Affymetrix) and Sanger
196   sequenced at Macrogen Inc (Netherlands) using both forward and reverse primers. Four
197   replicate PCRs per sample were sequenced to ensure consistency of sequence determination.
198   Sequences were assembled and aligned using known haplotypes as references (from Melo-
199   Ferreira *et al.*, 2012). A median-joining haplotype network (Bandelt *et al.*, 1999) was built
200   using Network v5.0.0.3 (www.fluxus-technology.com).

201

202   A combination of previously published sequence and genotype data (Alves *et al.*, 2008a;
203   Melo-Ferreira *et al.*, 2009; Melo-Ferreira *et al.*, 2012) was used to determine reference
204   variation on the five hare species distributed in Europe (*L. granatensis*, *L. europaeus*, *L.*
205   *castroviejoi*, *L. corsicanus* and *L. timidus*). Such reference SNPs were first chosen for having
206   a variant that was found fixed in a species while being absent in the others according to the
207   previous works. Second, given that some degree of variant sharing (though at low
208   frequencies) is expected due to hybridization (Melo-Ferreira *et al.*, 2009; Melo-Ferreira *et al.*,
209   2012), the first criterion was relaxed to include SNPs with variants that are predominant
210   ($\geq 75\%$) in one species and absent in the remaining or, in some rare instances, found at minor
211   frequencies in another species. Third, *L. castroviejoi* and *L. corsicanus* were often considered
212   together for the above criteria, as they are recently diverged sister species with a small
213   number of distinctive variants (Alves *et al.*, 2008a). These SNPs collectively provide
214   redundant information for species assignment. In addition, SNPs were selected to allow the
215   design of compatible primer pairs in the flanking regions to amplify a short ~10 bp fragment,
216   and for maximizing the inclusion of multiple informative SNPs in the amplicon. Thirty-two
217   PCRs organized in 8 multiplexes were performed (see PCR information in Table S1 in
218   Appendix S1) to genotype by sequencing a set of 41 informative SNPs on 12 autosomal genes
219   that followed the described criteria. The reference species variation and genotyped SNPs are
220   indicated in Table 2 and Table S2 in Appendix S1). One negative control was included in
221   each PCR.

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223   PCR products, including negative controls, were quantified with Qubit (Thermo Fisher
224   Scientific) and pooled in equimolar ratios. Individually barcoded libraries (with dual-index)
225   were prepared following Meyer & Kircher (2010) and Kircher *et al.* (2012). The six libraries
226   were sequenced using 100 bp single-end sequencing on 0.01% of a rapid run and 0.06% of a
227   high-output run lane on an Illumina HiSeq 1500 in CIBIO-InBIO's NEW-GEN sequencing
228   platform.

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230   Adapters were trimmed from raw sequence reads using Trimmomatic (Bolger *et al.*, 2014),
231   and reads mapped to reference sequences from the 12 nuclear genes, obtained from Melo-
232   Ferreira *et al.* (2012), using the bwa-mem algorithm (Li & Durbin, 2009). Samtools mpileup
233   and bcftools call (Li *et al.*, 2009) were used to infer SNPs with minimum base quality of 20
234   and dismissing alignments with mapping quality lower than 20. SNPs with sequence coverage
235   of a least 8x were retained using vcffilter, implemented in vcflib (Garrison, 2016).

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239 *2.3. Ecological niche modelling*

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241 Ecological niche modelling can provide the information needed to generate and test
242 hypotheses concerning the past structure of genetic diversity (e.g. Richards *et al.*, 2007),
243 supplying complementary information to that obtained from phylogeography (e.g. Diniz-Filho
244 *et al.*, 2013). In this context, we determined the macroecological requirements of the species
245 distribution at the present using an inductive approach (Corsi *et al.*, 2000). Data on species
246 distribution (presence/absence) were obtained at 50x50 UTM grid squares from The Atlas of
247 European Mammals (Mitchell-Jones *et al.*, 1999). This is the most complete dataset on the
248 distribution of species in Europe. In keeping with previous studies (Acevedo *et al.*, 2012a;
249 Acevedo *et al.*, 2017), analyses were restricted to Western Europe (see Figure 1), which
250 coincides with the most complete sampling areas identified by the authors.

251

252 Given that the aim of the models was to be transferred to a past environmental scenario, we
253 considered two macroecological factors, namely spatial and climatic factors, in a two-step
254 procedure. First, we delimited the geographical background by using the trend surface
255 analyses (TSA; spatial factor) following the procedure described in Acevedo *et al*. (2012b).
256 This procedure allows delimiting the adequate area to calibrate environmental models that,
257 when projected onto new scenarios, more reliably depict the potential distribution of the
258 species (Acevedo *et al.*, 2017). Once the geographical background was delimited for each
259 species, an environmental model was calibrated with climatic predictors (environmental
260 factor), using the WorldClim data base (Hijmans *et al.*, 2005).

261

262 Acevedo *et al.* (2015) tested seven modelling algorithms and the ensemble of their forecasts
263 when transferred to past climatic scenarios and showed that a Generalized Linear Model
264 (GLM) was able to produce robust estimates, with consistent predictions when compared to
265 the other methods. Accordingly, the models in this study, calibrated for the spatial or the
266 environmental factor, were performed using GLM, binomial distribution and logic link
267 function (Hosmer & Lemeshow, 1989). The environmental models were calibrated on a
268 random subset of the data (80%, calibration dataset) and the rest 20% was reserved for model
269 validation (validation dataset). First, a stepwise procedure based on AIC was used to select
270 the most parsimonious environmental models. Second, the predictive performance of the
271 models was assessed on the validation datasets in terms of discrimination, using the area
272 under the curve (AUC), and reliability, using calibration plots and Hosmer-Lemeshow test
273 (see Jimenez-Valverde *et al.*, 2013). Probabilities for species presence obtained from the
274 GLM were included in the favourability function (Real *et al.*, 2006) in order to obtain an
275 output independent of the species prevalence, the favourability (Acevedo & Real, 2012).

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277 The environmental models were then transferred to the past in order to create scenarios of
278 environmental favourability for the species. This procedure incorporates several sources of
279 uncertainty, in particular those related to different Global Circulation Models (GCMs),
280 characterized by different hypotheses on the effect of the oceans and atmosphere on climate
281 (Xu & Yan, 2001; Real *et al.*, 2010). We therefore transferred the models for the three hare
282 species (*Lepus granatensis*, *L. europaeus* and *L. timidus*) to Mid-Holocene, 6 kyr BP, using
283 three sets of climatic predictors, CNRM-CM5, CCSM4 and MIROC-ESM, downloaded from
284 http://www.worldclim.org/paleo-climate1. These models were chosen to broadly capture the
285 climatic variability found on most GCMs (e.g. Ramstein *et al*., 2007; Assis *et al*., 2018). The
286 transfers were done by replacing the values of the current bioclimatic predictors with those
287 estimated to past scenarios. The predictions in past scenarios were restricted to those
288 territories within the environmental domain of the environmental models according to

289   multivariate environmental similarity surfaces, MESS (Elith *et al.*, 2010). Finally,
290   favourabilities for species at 6 kyr BP were combined to infer, for each locality, the species
291   that achieved higher environmental favourability.

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295   **3. Results**

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297   *3.1. Molecular species assignment*

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299   A fragment of 360 bp of the mitochondrial genome was successfully sequenced in three
300   samples (SL007, SL012 and SL014), whereas for two samples (SL002 and SL005) 242 bp
301   were sequenced (GenBank accession numbers ###-###). The analysis of haplotype variation
302   unambiguously assigned the obtained haplotypes to *L. timidus* (Table 2; Figure S1 in
303   Appendix S1).

304
305   Of the 32 nuclear DNA amplified fragments encompassing 41 target SNPs, four
306   (corresponding to four SNPs) had sequence information in the negative control and were
307   therefore removed from further analyses. Of the remaining 37 SNPs, 27 were successfully
308   genotyped in at least one of five ancient DNA samples. The genotyping results are shown in
309   Table 2 and Table S2 in Appendix S1. For samples SL005, SL012 and SL014, four, two and
310   one SNPs, respectively, concerned variants that had been previously found fixed in *L.*
311   *granatensis* and absent in the other species (i.e. are diagnostic of the species). The remaining
312   genotyped SNPs in these samples are also compatible with *L. granatensis* reference variation.
313   For samples SL002 and SL007, the SPTBN1 variant is fixed in *L. granatensis* according to
314   our assessment of reference variation, but is also present in minor frequency in *L. castroviejoi*.
315   This sharing of variation had been shown to result from introgression from *L. granatensis* into
316   *L. castroviejoi* (Melo-Ferreira *et al.*, 2009) and therefore the SNP can be considered
317   diagnostic of *L. granatensis*. Again, the remaining genotyped SNPs in these samples
318   conforms to *L. granatensis* expectations. In sum, all five samples showed at least one
319   genotype that could unambiguously be attributed to *L. granatensis*. In all, the remaining
320   variation is compatible with *L. granatensis* and never diagnostic of another species (Table 2).
321   These result thus strongly suggest that the analysed autosomal variation assigns the samples to
322   *L. granatensis*, notwithstanding unknown possible levels of admixture with other Western
323   European hare species.

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326   *3.2. Niche modelling*

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328   The initial TSA models delimited the geographical background in which the environmental
329   models should be parameterized (Figure 1). The environmental models are summarized in
330   Table S3 in Appendix S1. For the three species, the models achieved a moderate-good
331   predictive performance in terms of both discrimination (*L. granatensis* AUC=0.77; *L.*
332   *europaeus* AUC=0.81; *L. timidus* AUC=0.88) and calibration (*L. granatensis* H-L: chi=9.22,
333   p=0.4; *L. europaeus* H-L: chi=41.4, p=0.004; *L. timidus* H-L: chi=81.8, p<0.001; see also
334   Figure S2), assessed using the validation datasets.

335
336   For the three analysed species, projections of species distributions to 6 kyr BP using three
337   different Global Circulation Models (CNRM-CM5, CCSM4 and MIROC-ESM) produced
338   strongly correlated results (Figure 1). The niche models suggested that 6 kyr BP most of

339   Europe was highly favourable to *L. granatensis*, but *L. europaeus* also showed strong
340   favourabilities in southern and western Europe, while the environmental conditions were not
341   favourable to *L. timidus* in southern Europe (Figure 1).

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345   Table 2: Summary of genetic variation from reference populations and inferred in the ancient
346   DNA (aDNA) samples.

Gene[1]	Pos.[2]	Reference species variation[3]					Variation in aDNA samples[4]				
		GRA	*EUR*	*CAS*	*COR*	*TIM*	SL002	SL005	SL007	SL012	SL014
CYTB	-	*gra/tim*	*eur/tim*	*tim*	*tim*	*tim*	*tim*	*tim*	*tim*	*tim*	*tim*
ALB	64	**A**	C	C	C	C	-	-	-	**A/A**	-
	65	G	G	**A/g**	**G/a**	G	-	-	-	G/G	-
	75	**A**	G	G	G	G	-	-	-	**A/A**	-
CA2	37	G	**T**	G	G	G	G/G	G/G	-	-	-
	43	T	**C**	T	T	T	T/T	T/T	-	-	-
	321	T/c	T	T	T	**C/t**	T/T	T/T	-	-	-
	421	G	G	**A**	**A**	G	-	G/G	-	-	-
	634	C	C	**T**	**T**	C	-	C/C	-	-	-
DARC	43	G	G	**A**	**A**	G	-	G/A	-	-	-
	404	**T**	C	C	C	C	-	C/C	-	-	-
	405	C	**T/c**	C	C	C	-	C/C	-	-	-
	417	**T**	G	G	G	G	-	**T/T**	-	-	-
HPX	121	C	C/a	C	C	**A/c**	C/C	-	-	-	-
	532	C	C	**T**	**T**	C	-	C/C	C/C	-	-
KITLG	270	**C**	T	T/c	T	**C/t**	-	C/C	-	-	-
SPTBN1	308	**G**	C	C/g	C	C	**G/G**	A/A	**G/G**	-	-
TSHB	110	A	**G/a**	A	A	A	-	-	-	-	A/A
OXA1L	49	T	T	T	**C**	T	T/T	T/T	-	-	T/T
	52	G	G	G	G	**A/g**	G/G	G/G	-	-	G/G
	58	A	A	A	A	**T/a**	A/A	A/A	-	-	A/A
UCP2	114	**T**	C	C	C	C	-	**T/T**	-	-	**T/T**
	270	A	A	**c/a**	**C**	A	-	A/A	-	-	-
	353	A	**G/a**	A	A	A	-	A/A	A/A	-	-
UCP4	58	**G**	T	T	T	T	-	**G/G**	-	-	-
	195	**G**	A	A	A	A	-	**G/G**	-	-	-
PPOX	440	g/c	C/g	C	C	**G/c**	-	C/C	-	-	-
	447	c/t	T/c	T	T	**C/t**	-	T/T	-	-	-

347
348   [1]Genes analysed in this study (see Table S1 in Appendix S1 for gene names).
349   [2]Coordinates of genotyped SNPs using alignments from Melo-Ferreira *et al.* (2012) as reference.
350   [3]Reference species variation according to previous works (Melo-Ferreira *et al.* 2009, 2012). GRA – *Lepus*
351   *granatensis*, EUR – *L. europaeus*, CAS – *L. castroviejoi*, COR – *L. corsicanus*, TIM – *L. timidus*. For CYTB
352   gene, origin of the haplotypes present in the species is indicated, reflecting introgression of *L. timidus* origin (see
353   Melo-Ferreira *et al.* 2005, 2012). The presumably fixed or nearly fixed variants are shown as single capital
354   letters. When polymorphic, a capital letter indicates the predominant allele (>=75%). The SNPs with strongest
355   diagnostic species information are underlined.
356   [4]Mitochondrial DNA haplotypes and autosomal genotypes inferred in this study. For CYTB, the species
357   assignment of the haplotype is shown (*tim* – *L. timidus*; *gra* – *L. granatensis*; *eur* – *L. europaeus*) (see Figure S1
358   in Appendix S1). Genotypes of the aDNA samples that are unambiguously attributed to *L. granatensis* are
359   underlined, and "-" indicates undetermined genotype.
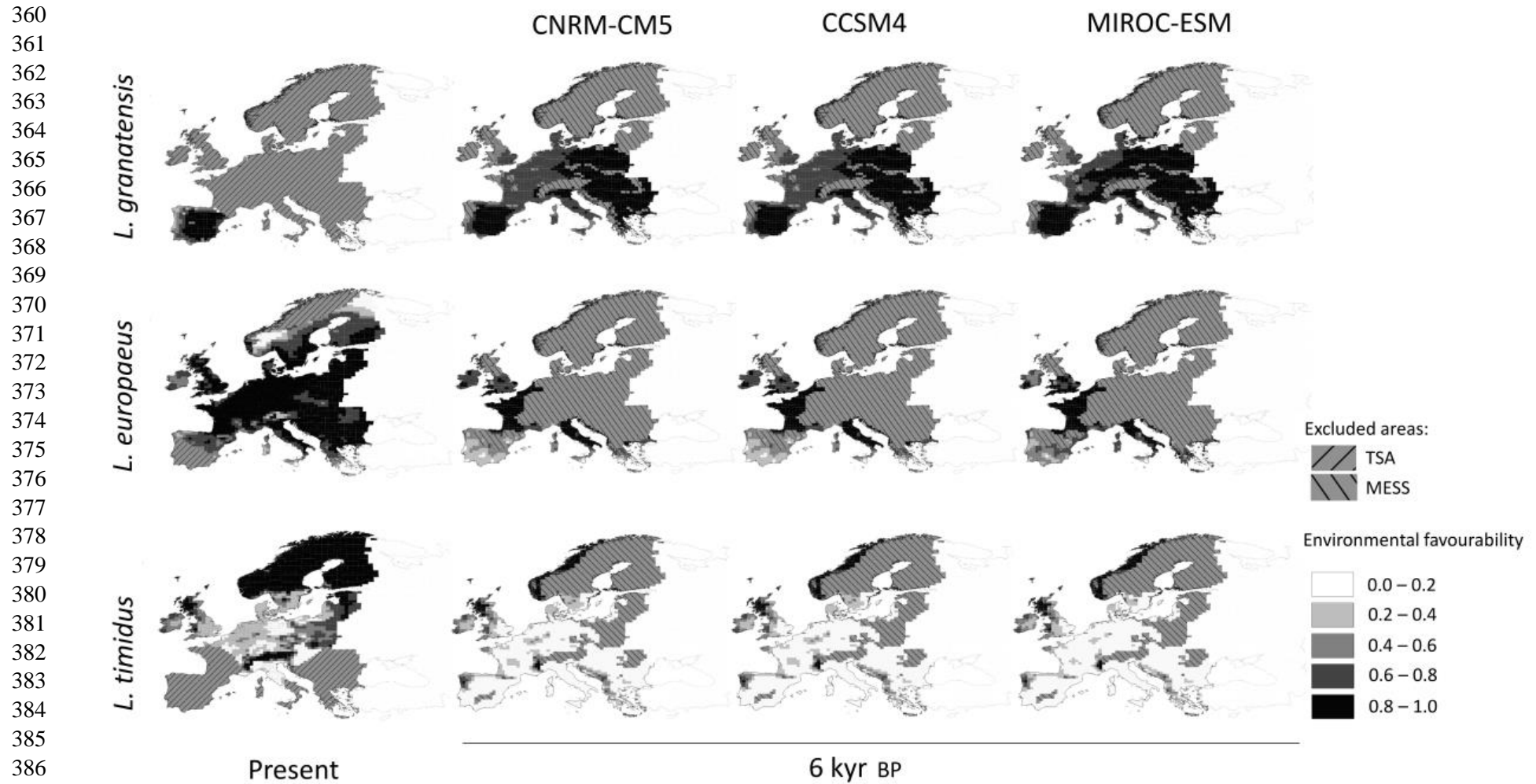
Figure 1: Favourability values for *Lepus granatensis*, *L. europaeus* and *L. timidus* presence for the present and 6 kyr BP in Western Europe using three Global Circulation Models (CNRM-CM5, CCSM4 and MIROC-ESM). Areas excluded in the trend surface analyses (TSA) and in multivariate environmental similarity surfaces (MESS) are indicated.

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392 Prior to transferring models to the past, MESS analyses identified areas included within the
393 environmental domain of each model, and the projection of models was then restricted to
394 these areas (Figure 1). Finally, the combination of favourabilities for species presence at 6 kyr
395 BP indicated to which species the climatic envelope was more favourable in a given locality.
396 Again, results were strongly consistent across tested Global Circulation Models (Figure 2).
397 While the Iberian Peninsula and southern France appeared more favourable to *L. granatensis*,
398 *L. europaeus* was favoured in the Italian Peninsula and western France, near the Pyrenees.
399 The screened bone samples were collected in sites with higher favourability to *L. granatensis*,
400 except one for the CNRM-CM5 Global Circulation Model (Paulhan), where the environment
401 of the 50x50 Km square appeared more favourable to *L. europaeus* (Figure 2).
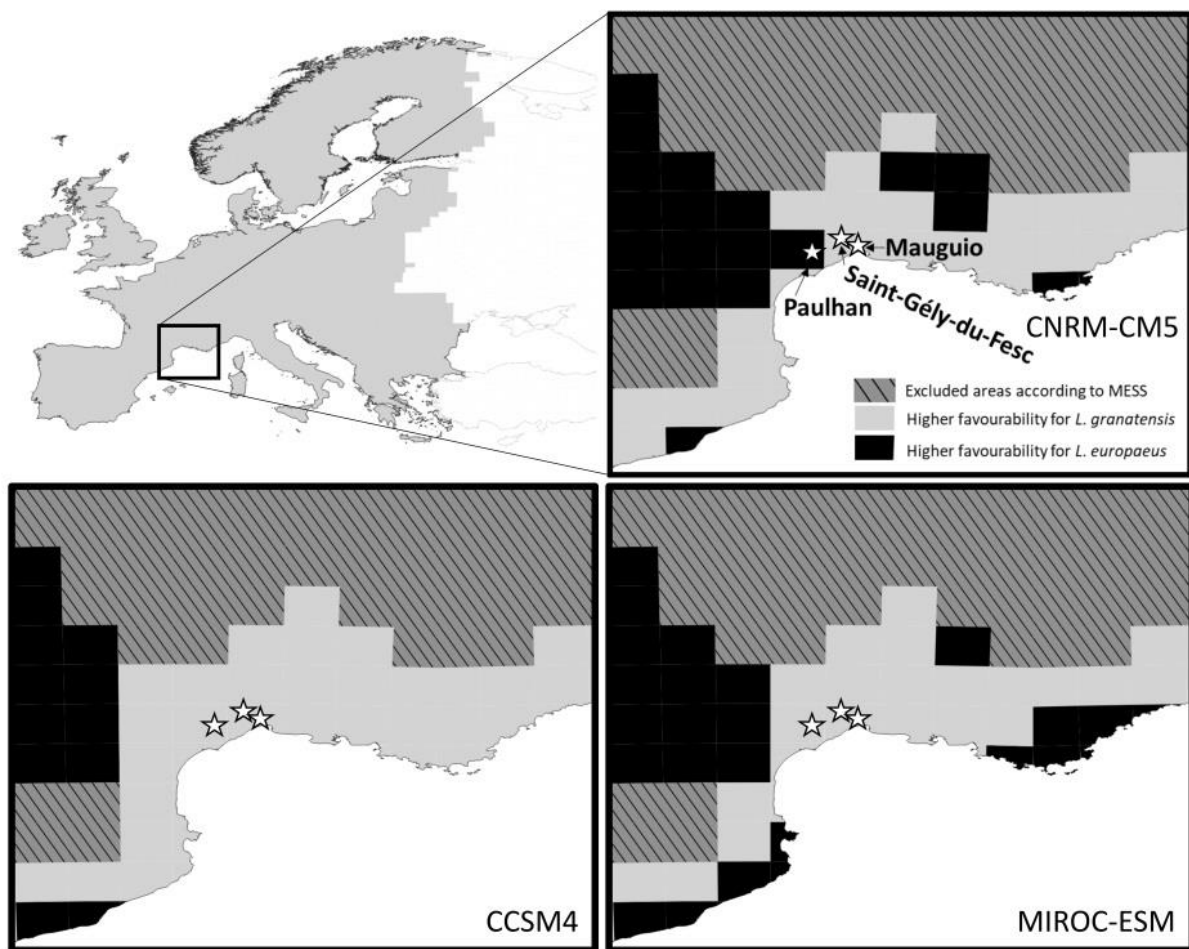
434 **Figure 2**: Map of Western Europe showing the species with higher environmental
435 favourability in the sampling localities 6 kyr BP using three Global Circulation Models
436 (CNRM-CM5, CCSM4 and MIROC-ESM). Areas excluded in the multivariate environmental
437 similarity surfaces (MESS) are shown. Stars depict sampling localities.

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4. Discussion

Ancient DNA provides powerful means to identify species present in a given region at a certain time and is therefore valuable to understand the past distribution of species, and consequently characterize range changes. This is particularly relevant for closely related species with important overlap of morphological characters, which make the precise species diagnosis solely based on bone morphology non-trivial, as is the case of hares (Angermann, 1983). Our ancient DNA analysis clearly showed that all five samples have genetic variants that are exclusive of *L. granatensis* (Table 2), a species presently confined to the Iberian Peninsula. This result agrees with the described morphology, as *L. granatensis* is the smallest hare species in Europe (Palacios, 1989). The presence of this species in southern France 5.5-7 kyr BP could result either from human-mediated introductions or natural colonization from Iberia, where the species was likely restricted at the LGM (see Acevedo *et al.*, 2012c). Our ecological niche models confirm that the climatic characteristics of the region were generally favourable to *L. granatensis* 6 kyr BP (Figures 1 and 2), which therefore suggests that the species could have reached southern France due to natural expansion, out of Iberia, but subsequently retracted its range. Interestingly, all five analysed ancient samples possess the mtDNA haplotype from *L. timidus* (Table 2; Figure S1 in Appendix S1). This mtDNA type is the most frequent in the northern range of *L. granatensis*, which results from ancient introgression (Melo-Ferreira *et al.*, 2005; Melo-Ferreira *et al.*, 2012), during the range replacement of *L. timidus* in northern Iberia (Acevedo *et al.*, 2015; Marques *et al.*, 2017; Seixas *et al.*, 2018). Our results thus suggest that the postglacial northwards expansion of *L. granatensis* extended beyond the species' current range, through the Pyrenees, and reached Southern France 6 kyr BP.

The niche models show that the environment in Western Europe 6 kyr BP also favoured *L. europaeus*, including at one of our sampling sites for one of the studied GCMs (Figure 2). Phylogeographic analyses of *L. europaeus* suggest that Western Europe may have been colonized from a source population located in central/south-central Balkans that started expanding at late Pleistocene or early Holocene (Stamatis *et al.*, 2009). That Western Europe was colonized recently and no glacial refugium for the species existed in the region is also supported by the lower levels of genetic variation found in the species in Western European populations. The interpretation of the paleontological records suggests that the unequivocal presence of *L. europaeus* in Southern France can only be attested at the end of the Neolithic (4.4 kyr BP), after which it became abundant (Forest & Cheylan, 2015). Conflicting reports suggesting that the species may have been present in Southern France earlier had difficulties separating bones of *L. europaeus* and *L. timidus* (e.g. Pascal & Vigne, 2003; see also Forest & Cheylan, 2015), and did not take into account the possible presence of *L. granatensis*. Future molecular-based species assignment of bone remains, as we do in this work, may help clarifying this issue. A late arrival of *L. europaeus* to Southern France, after the date explored in our analysis (6 kyr BP) despite general habitat favourability (Figure 1), may be explained by several factors that we cannot evaluate at this point, such as colonization rate, competition with other species, ability to transpose barriers or opportunity. Nevertheless, the environmental conditions in Western Europe may have acted as a pole of attraction for the species, and interspecific competition may have contributed to the range contraction of *L. granatensis* back to an exclusive Iberian range. Such competitive replacement hypothesis is supported by the general tendency of hare species to exclude each other and avoid sympatry, with only transient and minor areas of co-occurrence (Flux, 1981). Broad distributions of hare species are generally either allopatric or parapatric with little overlap, and that is the case in Western European hares (Thulin, 2003), suggesting that interspecific competition is a major

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492   determinant of the distribution boundaries (Leach *et al.*, 2015). In the current contact between
493   *L. europaeus* and *L. granatensis* in Northern Iberian Peninsula, densities are smaller in the
494   narrow area of co-existence of the species, and asymmetric species competition has been
495   suggested (Gortazar *et al.* 2007; Acevedo *et al.*, 2012a). An ecological and demographic
496   advantage of *L. europaeus*, typical of Continental and Atlantic climates, over the
497   Mediterranean *L. granatensis* during the westwards invasion of the former would thus explain
498   the retraction of *L. granatensis* back to Iberia, mimicking, in the reverse sense, the postglacial
499   range replacement of *L. timidus* by *L. granatensis* in northern Iberia (Acevedo *et al.*, 2015;
500   Seixas *et al.*, 2018).

501

502   Our niche models and the finding of *L. granatensis* in southern France make it less likely that
503   the cold-adapted *L. timidus* could have remained in the region until the arrival of *L. europaeus*
504   (Figure 1). The replaced *L. granatensis* populations carried mtDNA haplotypes of *L. timidus*
505   origin, which subsequently introgressed into *L. europaeus*, leading to the high frequencies of
506   mtDNA introgression found nowadays in the species in its Iberian range (Melo-Ferreira *et al.*,
507   2005). Remarkably, despite successive species invasions, the *L. timidus* mtDNA haplotypes
508   found in northern Iberia mark the historical distribution of the species (the "home-loving"
509   mtDNA, Melo-Ferreira *et al.*, 2014; see also Acevedo *et al.*, 2015), due to repeated mtDNA
510   introgression. Introgression from a resident into an invading species is expected to be
511   favoured in situations of species range replacements with hybridization, due to repeated
512   hybridization and the demographic imbalance in the invasion front, where the densities of the
513   invading species is lower (Excoffier *et al.*, 2009). This effect is predicted to lead to
514   preferential mtDNA introgression in species with lower female migration rates, as is often the
515   case in mammals, due to lower inputs of native female-linked variants into the invasion front
516   (Currat *et al.*, 2008), and in situations of asymmetrical introgression, as inferred in the *L.*
517   *timidus-L. granatensis* system in the Iberian Peninsula (Seixas *et al.*, 2018). Recurrent range
518   replacements through climate oscillations may thus lead to recurrent introgression among
519   closely related hybridizing species.

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521   The results presented here provide paleontological and ecological evidence that the current
522   ranges of hares in South-western Europe result from at least two sequential postglacial range
523   replacements (*L. timidus* by *L. granatensis* and then *L. granatensis* by *L. europaeus*). Climatic
524   oscillations not only induced range revolutions, but also affected the genetic composition of
525   these natural populations in areas transiently occupied by closely related species, due to
526   introgressive hybridization. The patterns of introgression are striking for mtDNA but
527   hybridization also affected the nuclear genome (Melo-Ferreira *et al.*, 2009; Melo-Ferreira *et*
528   *al.*, 2011), sometimes massively (Seixas *et al.*, 2018). Southern Europe was therefore an area
529   of active evolution via genetic exchange for hare species, with potential introgression of
530   neutral variants, purging of genetic incompatibilities and local natural selection (Seixas *et al.*,
531   2018). Contractions and expansion of species, often with replacements of ranges of closely
532   related hybridizing species, is a ubiquitous phenomenon that occurred recurrently in the
533   history of many taxa as a consequence of climatic changes. These dynamics have important
534   implications in current biogeographic patterns and in the genetic composition of natural
535   populations (e.g. Excoffier *et al.*, 2009). Thus, inferring the complex dynamics of past range
536   changes and interspecific interactions is fundamental to understand current distributions of
537   biodiversity at different scales, from the species level to the organization of genetic variation.

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542   **Data accessibility**

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544   Mitochondrial DNA sequences are deposited in GenBank with accession numbers
545   MH800846-MH800850.

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548   **References**

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740 **Biosketch**

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742 Sara Lado is an Evolutionary Biologist interested in phylogeography, systematics and
743 conservation, with special interest in African mammals. She obtained her Master degree at
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748 **Author contribution**

749

750 JMF devised and coordinated the work with contributions from LD, PA and VF. VF provided
751 the bone samples. SL, LF and LD performed the laboratory work. JMF performed the genetic
752 analysis. PA performed the ecological modelling. JMF wrote the paper with contributions
753 from the other authors. All authors interpreted the data, and read, revised and approved the
754 final version of the manuscript.

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