INTERSPECIFIC X-CHROMOSOME AND MITOCHONDRIAL DNA INTROGRESSION IN THE IBERIAN HARE: SELECTION OR ALLELE SURFING?

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Introgression from a resident species into an invading one is predicted to occur through the demographic process of "allele surfing," and to particularly affect genomic regions transmitted by the lower migrating sex, such as mtDNA. This could explain that northern Iberian populations of *Lepus granatensis* harbor high frequencies of mtDNA from *L. timidus*, an arctic hare it replaced there after deglaciation. We report that variation of introgressed *timidus*-like mtDNA reflects several predicted effects of this process: increasing frequency and diversity in the direction of expansion, strong perpendicular phylogeographic structure and signs of postglacial demographic growth. However, demographic inferences for the *granatensis* and *timidus*-like mtDNA lineages suggest the latter may have outcompeted the former in northern Iberia. Autosomal introgression occurs at low frequencies and species-wide rather than only in the north. If this difference with mtDNA resulted from sex-biased migration, an intermediate pattern should prevail for the X-chromosome, but we report species-wide and high-frequency introgression of an X-fragment. Either selection favored this ubiquitous X-introgression, or more complex postglacial expansion patterns prevailed, with different consequences depending on the genomic and geographic region. This illustrates the difficulty of distinguishing demographic and selective effects and the need for genome and species-wide based demographic models.

KEY WORDS: ***Lepus granatensis*, *Lepus timidus*, invasion, hybridization, glaciation.**

Introgressive hybridization between differentiated populations, subspecies, or species is often reported and can be viewed as a nuisance complicating the use of genetic markers for systematics and biodiversity inventorying, but provides interesting situations for evolutionary studies (Arnold 2006). Because introgression is generally asymmetrical, often massive and classically highly variable among genomic regions, the question of its potential adaptive nature is often raised (Currat et al. 2008; Petit and Excoffier 2009 e.g., for recent review of some cases). However, theory predicts that in situations of range expansion of one species into that of another, all these characteristics could result from mostly stochastic demographic processes, in conjunction with variations of modes of transmission among genomic regions. Because populations at the front of an expansion wave are at low densities, genetic drift can easily bring rare alleles to high frequencies and subsequently propagate them during demographic expansion and further progression of the wave. Thus foreign alleles introduced by hybridization into the front populations of the invading species,

even if initially rare when hybridization is such, could "surf" on the wave of expansion with high probability and reach high frequencies beyond the initial contact between species. Intraspecific gene flow from the parental populations of the expanding species should however tend to dilute and erase such traces of introgression, but the efficiency of this process is expected to vary among genomic regions depending on their mode of transmission, because migration is often sex-biased (Klopfstein et al. 2006; Currat et al. 2008; Excoffier and Ray 2008; Excoffier et al. 2009). Literature surveys have argued that many cases of massive introgression could correspond to such situations of range expansion, and that patterns of differential introgression between mtDNA, autosomes, and sex chromosomes generally conform to the predictions that can be made on the basis of their mode of transmission and on which sex disperses more (Currat et al. 2008; Petit and Excoffier 2009).

This theoretical framework offers an interesting null hypothesis to study introgression and question the potential role of selection in its determination. Case studies have yet rarely been investigated in such a hypothetical frame, and the Iberian hare (*Lepus granatensis*) offers a good model to do so: among populations of this endemic species present over most of Iberia, those from the northern half of the Peninsula harbor high frequencies of mtDNA from another species, *L. timidus* (Melo-Ferreira et al. 2005; Melo-Ferreira et al. 2007; Alves et al. 2008). Although the latter, arctic/boreal, species is presently not found in Iberia, these observations attest of its ancient presence there, at least until the end of the last glaciation, according to paleontological data (Altuna 1970). It is therefore logical to suppose that *L. granatensis* expanded from southern to northern Iberia, into a territory occupied by *L. timidus,* at the favor of climate warming during deglaciation (Melo-Ferreira et al. 2007), and that this could account for the observed mtDNA introgression pattern. A study of 10 autosomal genes provided evidence of introgression in the same direction, from *L. timidus* into *L. granatensis* (Melo-Ferreira et al. 2009). Contrary to that of mtDNA, autosomal introgression was at very low frequency, and spread all over Iberia rather than restricted to the north. Within the theoretical framework depicted above this could tentatively be explained by higher male migration in *L. granatensis*, which would have diluted initial introgression of bisexually transmitted autosomes to a greater extent than for the female transmitted mtDNA, but could also have favored later diffusion of rare introgressed alleles to the rest of the species. If such was the case, one could predict an intermediate pattern for the X chromosome, which is transmitted more often by females than males (twice more if sex ratio is balanced). However, such is not the case. Two centromeric X markers that were tested showed no introgression at all (Melo-Ferreira et al. 2009). Here, we tested a telomeric X marker and found the most extensive introgression of all nuclear markers analyzed to date, both in frequency and geographic range. The centromeric X result could be explained by selection against hybridization, because the X is known to often harbor hybrid male incompatibility factors, which is corroborated by the absence of Y chromosome introgression (Melo-Ferreira et al. 2009). However the telomeric X result appears difficult to reconcile with the general historical and geographical scenario elaborated on the basis of mtDNA and theoretical predictions. Therefore here we also analyze species-wide mtDNA sequence variation of *granatensis* and *timidus*-like lineages in the light of this hypothesis and discuss possible alternative geographic and selective scenarios that could account for the contrasted introgression patterns between genomic regions.

Materials and Methods

LABORATORY METHODS

We amplified a mitochondrial cytochrome *b* (CYTB) fragment using primers LCYF (Alves et al. 2003) and LCYTBR (Melo-Ferreira et al. 2005), and a control region (CR) fragment using primers LCRSEQ (Melo-Ferreira et al. 2007) and LepD2H (Pierpaoli et al. 1999) in 15 *L. granatensis* specimens known to harbor the *timidus* mtDNA lineage and 234 with the native *granatensis* lineage (assessed following Melo-Ferreira et al. 2005).

Maleness of 69 *L. granatensis* and 17 *L. timidus* was assessed by the successful amplification of the SRY gene (Melo-Ferreira et al. 2009). For these 86 specimens, we amplified a fragment including part of intron 2 of the X-linked PHKA2 gene (Phosphorylase Kinase alpha 2) using primers NPhka2e2f (Geraldes et al. 2006) and LPhka2i23r1 (5′-AAAGGGGGATGGGAAATGT G-3′).

The mtDNA PCR products were sequenced using primers LCYF and LCRSEQ whereas for PHKA2 we used the forward PCR primer and the reverse primer LPhka2i23r2 (5′-GCACTTGTGTAAAAGCCATTCAG-3′). We followed the ABI PRISM BigDye Terminator Cycle Sequencing 3.1 standard protocol and performed electrophoresis on an ABI PRISM 3130 sequencer.

SEQUENCE DATA ANALYSES

The mtDNA data obtained in this work (249 sequences of CYTB and CR) were aligned together with previously published sequences (Melo-Ferreira et al. 2007) obtained from 180 *L. granatensis* affected by mtDNA introgression from *L. timidus* (GenBank Accession Nos. DQ882960-DQ882985, DQ882988-DQ883026, DQ883129-DQ883154, and DQ883157-DQ883195), for which we provide here the detailed geographic origin (Tables 1 and S3).

DnaSP 4.20 (Rozas et al. 2003) was used to identify haplotypes both in the mtDNA and X-chromosome datasets. The relationships among the X chromosome and among *granatensis*-like and *timidus*-like mtDNA haplotypes were determined by

Table 1. Localities of *L. granatensis* (*gra*) and *L. timidus* (*tim*) sampled, with the number of specimens sequenced for mtDNA (with distribution among origins and sublineages) and for the PHKA2 locus (X).

Sp	Number	Code	Locality	*granatensis*		*timidus*			mtDNA*	X
				nA	nB	iA	iB	iC	*n*	*n*
gra	1	Alj	Aljustrel		**10**				10	**3**
	2	Cac	Caceres	**8**	**2**				10	
	3	CBr	Castelo Branco		**10**				10	
	4	Crd	Cordoba	**1**	**4**				5	**1**
	5	CRe	Ciudad Real		**10**				10	**3**
	6	Grn	Granada		**10**				10	**6**
	7	Mnt	Montalegre		**8**				8	
	8	Pan	Pancas		**10**				10	**5**
	9	Ptm	Portimão		**10**				10	**2**
	10	Sev	Sevilla	**1**	**7**				8	**3**
	11	Ala	Álava		**10**	13			23	**5**
	12	Alb	Albacete		**10**		1		11	**5**
	13	Alc	Alcañiz		**5**	1	18		24	**4**
	14	Ali	Alicante		**10**	2			12	**4**
	15	Amd	Almeida		**8**			**5**	13	
	16	Ben	Benavente	**1**	**3**	21			25	**2**
	17	Brg	Bragança		**2**	8			10	
	18	Cue	Cuenca		**10**		1		11	**6**
	19	FCR	Figueira Castelo Rodrigo		**4**			**5**	9	
	20	Mad	Madrid		**10**	6	8		24	**5**
	21	Nav	Navarra		**1**	6	16		23	**2**
	22	Sal	Salamanca		**10**	**2**		**1**	13	
	23	SE	Serra da Estrela		**9**			**2**	11	
	24	Sen	Sendim	**3**	**7**	1		2	13	
	25	Sor	Soria		**5**	3	17		25	**4**
	26	TC	Tierra de Campos	**1**	**8**	13			22	**4**
	27	Tol	Toledo		**10**	7			17	**3**
	28	Tor	Tordesillas		**10**	5			15	
	29	Val	Valencia		**4**		1		5	
	30	Zar	Zaragoza		**2**	12	18		32	**2**
tim	31	Swe	Sweden							**6**
	32	Alp	Alps							**4**
	33	Nor	Norway							**2**
	34	Ura	Urals							**1**
	35	FER	Far East Russia							**4**
	TOTAL			15	219	100	80	15	429	86

***Data obtained in the present work are in bold face. Previously available data: GenBank Accession Nos. DQ882960-DQ882985', DQ882988-DQ883026', DQ883129-DQ883154, and DQ883157-DQ883195, see also Melo-Ferreira et al. (2007).**

constructing median-joining networks (Bandelt et al. 1999) using Network 4.5 (www.fluxus-technology.com). Arlequin 3.0 (Excoffier et al. 2005) was used to calculate the various descriptive diversity statistics, to perform tests of conformation to equilibrium and neutrality (significance tested by 10,000 simulated datasets), to conduct analysis of molecular variance, and to fit mismatch distributions to the sudden expansion model (confidence intervals on parameters determined by 1,000 bootstraps). To examine the spatial structure of mtDNA variation, a spatial analysis of molecular variance (SAMOVA, Dupanloup et al. 2002) was also conducted.

Demographic inferences using mtDNA were also performed based on the coalescent, using Fluctuate 1.4 (Kuhner et al. 1998). The final estimates resulted from a run of 10 short chains of 1000 steps followed by 10 long chains of 20,000 steps, sampling every 10 steps. Because the estimates of the growth parameter g are known to be biased upwards (Kuhner et al. 1998), we followed the conservative method used by Lessa et al. (2003) and considered

g to indicate population growth only if $g > 3$[SD] and population decline if $g < -3$[SD]. In addition, demographic history was inferred using the Bayesian skyline plot (BSP) methodology (Drummond et al. 2005) as implemented in the BEAST software. To overcome computer power limitations, three sets of 100 random sequences were picked for each of the analyzed mtDNA datasets. Estimates were based on genealogies obtained using the MCMC sampling procedure, using the strict clock assumption, and setting the number of discrete intervals to 20. Three runs of 25 million iterations, of which the first 10% were discarded as burn-in, were performed for each randomly created dataset to ensure the consistency of the estimates. The model parameters were sampled every 2500 iterations. We used the Hasegawa–Kishino–Yano (HKY) model of mutation and estimated the gamma distribution parameter and the transition/transversion ratio together with the BSP parameters independently for CYTB first, second, and third codon positions and for CR. The BEAST resulting log and tree files were analyzed using Tracer version 1.5 (A. Rambaut & A. Drummond, http://evolve.zoo.ox.ac.uk/software.html) and TreeAnnotator version 1.5.4 included in the BEAST package, estimating the BSP and time to the most recent common ancestor (TMRCA), respectively.

All analyses based on mtDNA in *L. granatensis* were performed separately on the *timidus*-like and *granatensis*-like lineages, despite their co-occurrence in some of the populations. Doing so precludes using the results of the analyses to draw conclusions or infer population genetics parameters relating to the demography of the populations where they were sampled. However because mtDNA evolves without recombination, analyzing the coalescent separately on the two lineages provides insight into the evolution of these lineages.

To detect recombination at the X locus, the Maximum chi-square statistic was calculated as implemented in RecombiTEST (www.lifesci.sussex.ac.uk/CSE/test; Piganeau et al. 2004).

Results

VARIATION IN THE *TIMIDUS*-LIKE mtDNA LINEAGE

We sequenced fragments of the mtDNA cytochrome b gene and of the CR (617 and 472–474 bp respectively) in 15 *L. granatensis* (GenBank Accession cytb: JF298923-JF298926; CR: JF299044-JF299047) that were known to harbor mtDNA of *L. timidus* origin from a preliminary PCR-RFLP assay following Melo-Ferreira et al. (2005) (Tables 1 and S3 for sampling details). These data were put together with previously published sequences (Melo-Ferreira et al. 2007) sampled from 180 *L. granatensis* specimens affected by mtDNA introgression from *L. timidus* (130 haplotypes). Substantial nucleotide variation was found (Table 2), which as shown on the network of Figure S1 resulted from the existence of three divergent lineages, that we named iA, iB, and iC. On the map of Fig. 1A (details in Table 1) one can see that the three lineages are clearly geographically separated along an east–west direction. This is reflected in the results of the SAMOVA: incrementing the number of population groups (K) gave the most pronounced increase of intergroup differentiation for $K = 3$. The geographic envelopes of these three population groups (that we named I1-I3) are delineated on Fig. 1A, and Table 3 shows that in

Table 2. Sequence diversity, neutrality tests, growth rate estimates and mismatch distribution analyses in *L. granatensis* carrying the *granatensis* and *timidus*-like mtDNA haplotypes.

Groups	*n*i	Polymorphism			Neutrality		growth (g)	Mismatch analysis‡		
		*n*h	*h* (%)	π (%)	Tajima's *D*	Fu's *Fs*		τ	θ_0	θ_1
Haplotypes in *L. granatensis*										
Total	429	186	99.2 (0.1)	6.2 (3.0)	2.03	−17.60	65.63 (8.3)†	–	–	–
granatensis-like haplotypes										
Total	234	117	98.9 (1.6)	1.4 (0.7)	−1.39	−23.79*	231.1 (23.3)†	12.0	4.1	139.3
Pure pop.	91	49	97.8 (0.5)	1.4 (0.7)	−1.10	−12.24*	182.5 (31.3)†	10.7	6.8	86.1
Mixed pop.	143	68	98.0 (0.4)	1.3 (0.6)	−1.01	−23.62*	173.1 (35.8)†	13.5	2.4	78.8
Lineage nA	14	11	95.6 (4.5)	0.9 (0.5)	−0.43	−1.32	458.5 (92.7)†	12.4	0.0	95.8
Lineage nB	220	106	98.9 (1.7)	1.2 (0.6)	−1.45*	−23.89*	249.2 (36.5)†	13.4	1.5	101.1
timidus-like haplotypes										
Total	195	69	97.8 (0.3)	1.9 (0.9)	0.43	−7.86	118.0 (38.3)†	–	–	–
Lineage iA	100	30	95.7 (0.7)	0.6 (0.3)	−0.87	−6.08	285.5 (110.0)	5.80	2.00	32.4
Lineage iB	80	33	94.6 (1.3)	0.6 (0.3)	−1.30	−12.07*	316.4 (56.2)†	6.00	1.40	18.6
Lineage iC	15	6	76.1 (9.6)	0.4 (0.3)	−0.94	1.67	−61.7 (103.1)	7.40	0.00	4.1

ni = number of analyzed individuals; nh = number of observed mtDNA haplotypes; h = haplotype diversity; π = nucleotide diversity, g = growth parameter. Standard deviations are shown in brackets and † indicates g > 3SD. The significant values are indicated by an asterisk. ‡In the mismatch analyses values are only shown when the sudden expansion model was not rejected.

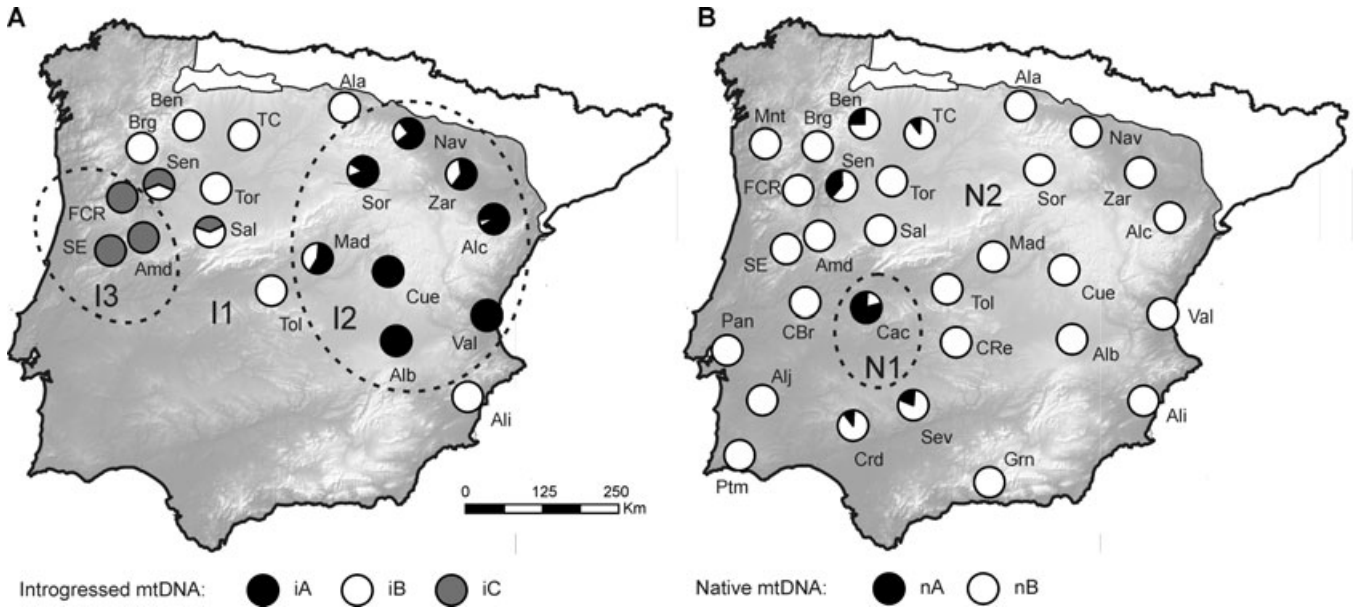

Figure 1. **Frequencies of mtDNA sublineages in *L. granatensis*. (A) Haplotypes of *timidus* origin (sublineages iA, iB, and iC), and (B) native *L. granatensis* haplotypes (groups nA and nB). The gray shading indicates topography (the lighter the more elevated). The groups of populations identified by the SAMOVA analysis are indicated: I1-I3 in (A) and N1-N2 in (B).**

this partition, most of the molecular variance (54.55%) lies among groups, but little among populations within the groups (6.28%). A significant south–north increase was detected for haplotype diversity (π, Pearson correlation, $P = 0.006$) but not for nucleotide diversity (*h,* Pearson correlation, $P = 0.422$; data from Table S2). Considering the three lineages separately, we found increasing diversity to the north for π and *h* in lineage iA (Pearson correlation, $P = 0.029$ and $P = 0.006$ respectively) but not in iB (iC has a very restricted range, so the calculations could not be performed).

VARIATION IN THE *GRANATENSIS*-LIKE mtDNA LINEAGE

We sequenced the same two mtDNA fragments in a species-wide sample of *L. granatensis* with mtDNA of *granatensis* origin as assessed following Melo-Ferreira et al. (2005) (234 specimens from 30 locations, Table 1. GenBank Accession Numbers, cytb: JF298927-JF299043; CR: JF299048-JF299164). Haplotype and nucleotide diversities were of the same order as among the haplotypes of *timidus* origin (Table 2). The haplotype network displayed little structure, apart from the existence of two rather divergent lineages nA and nB depicted in Figure S1B. Group nA was much rarer and predominantly occurred in one population. Accordingly, the SAMOVA analysis with $K = 2$ separated this population from all the others (these two groups of populations were designated N1 and N2; Fig. 1B) and this was the level of partition that increased most intergroup variance. However, only a quarter of the detected variance was attributable to this intergroup differentiation (Table 3). The I1-I3 population groups that

Table 3. **Analysis of molecular variance of mtDNA on several partitions of the sampled populations.**

Groups of populations	Fixation indexes			Percentage of variation		
	Φ_{SC}	Φ_{ST}	Φ_{CT}	AG	APWG	WP
granatensis-like haplotypes in *L. granatensis*						
All populations	–	0.462	–	–	46.22	53.78
N1/N2*	0.447	0.588	0.256	25.60	33.22	41.18
I1/I2/I3*	0.408	0.492	0.143	14.27	34.98	50.75
timidus-like haplotypes in *L. granatensis*						
All populations	–	0.497	–	–	49.67	50.33
I1/I2/I3*	0.138	0.608	0.546	54.55	6.28	39.17

*See Figure 1 for the composition of each group. AG = among groups; APWG = among populations within groups; WP = within populations.

best partitioned the molecular variance of the *timidus* haplotypes reveal little differentiation based on the *granatensis* haplotypes in the same populations (14.27% of the variance as opposed to 54.55% when considering the *timidus* haplotypes; Table 3). Overall, when the major subdivisions are taken into account (I1-I3 for *timidus* mtDNA and N1-N2 for *granatensis*), population structure appears more pronounced for the *granatensis* than for the *timidus* lineage ($\Phi_{SC} = 0.447$ vs. 0.138).

Contrary to what we found with the *timidus* lineage, there was no correlation between latitude and diversity in the *granatensis* lineage, whether considering all populations (π: $r^2 = 0.04$, $P = 0.374$; h: $r^2 = 0.08$, $P = 0.163$), the mixed populations, that is, populations harboring both *granatensis* and *timidus*-like mtDNA (π: $r^2 = 0.01$, $P = 0.688$; h: $r^2 = 0.12$, $P = 0.196$), or the pure populations, with only *granatensis*-like mtDNA (π: $r^2 = 0.01$, $P = 0.847$; h: $r^2 = 0.01$, $P = 0.789$; data in Table S2).

DEMOGRAPHIC INFERENCES BASED ON MTDNA

As expected given the structure of the *timidus* haplotype network (Fig. S1), the mtDNA data of *timidus* origin present in *L. granatensis* produced a multimodal mismatch distribution (Fig. S2B), and the sudden expansion model was rejected (Table 2). However the coalescent-based approach gave indications of growth, with a high positive *g* parameter (Table 2). Because the three haplogroups are geographically separated, we considered valid to further analyze each of them separately. Lineage iC had low sample size and yielded contradictory results, but for the two other lineages, all tests were compatible with a history of expansion, and significantly so for parameters *Fs* and *g* in the case of lineage iB. Similar results were obtained when considering population groups I1–I3 instead of the three lineages (not shown).

Similar analyses based on the *granatensis* haplotypes yielded homogeneous and coherent results, whether on the whole sample or after distinguishing pure populations (harboring only *granatensis*-like mtDNA, in the south) and mixed populations (harboring both *timidus* and *granatensis*-like mtDNA, in the north). The unimodal mismatch distribution (Fig. S2A) fitted the expectation under the sudden expansion model, and the growth parameter *g* and *Fs* statistics were concordant (respectively positive and negative) and generally significant (Table 2). The estimated date of the expansion was however consistently older for the *granatensis* haplotypes than for those of *timidus* origin.

We also applied the Bayesian Skyline Plot method by considering separately different datasets and the results are presented in Figure 2. When considering only haplotypes of *timidus* origin, the method infers a relatively recent demographic growth. When considering haplotypes of *granatensis* origin, the results differ between the mixed and pure populations. Both display a signal of relatively old expansion, but their skylines differ for the more recent period corresponding roughly to the period of inferred growth of the *timidus* haplotypes. Although the analysis suggests that growth continued in the pure populations during that time, it suggests on the contrary population size reduction in the geographic region where the *timidus* lineage is also found. These results must however be considered with extreme caution because confidence intervals are very wide (Fig. S3) and when taking them into account, none of the trends described above appears sustained with confidence. However despite these wide confidence intervals, the correlation between the results obtained on these three independent datasets is remarkable and worth considering. The suggested recent growth inferred for the *granatensis* lineage in pure populations and of the *timidus* lineage in mixed populations is in line with the idea that climatic conditions recently became more favorable to *L. granatensis*, allowing it to prosper. However the analysis suggests that the *granatensis* mtDNA lineage declined in the north rather than prospering as the *timidus* mtDNA lineage appears to have done.

X-CHROMOSOME VARIATION

We sequenced a 1058–1068 bp fragment of the PHKA2 gene, known to be distal on the rabbit X chromosome (Geraldes et al. 2006) in 86 males from *L. granatensis* and *L. timidus* (GenBank Accession Numbers JF299165-JF299183. Note that some of the haplotypes described in the present study appear under several accession numbers because of short insertion/deletion differences that were not considered in the present analyses). In both cases sampling covered most of the species ranges (Table 1 and the map of Fig. 4B for the geographic location of *L. granatensis* samples). Intraspecific nucleotide diversity was higher in *L. granatensis* (0.38%, $N = 69$) than in *L. timidus* (0.14%, $N = 17$). In the network of Fig. 3A (Table S4 gives the geographic distribution of haplotypes) *L. granatensis* haplotypes (designated by the letter G) can be assigned to two haplogroups that we named 1 and 2 and are separated by four mutations all lying in the 5′ portion of the fragment (positions 21 to 435). Therefore in this proximal segment, *L. granatensis* haplogroup 2 is more similar to *L. timidus* (haplotypes designated with letter T) than to haplogroup 1, which could result from a recombination between haplotypes of *L. granatensis* and *L. timidus* origins. The maximum Chi-square test (Piganeau et al. 2004) detected significant signs of such recombination between sites 435 and 471 when considering the haplotypes of both species together (MaxChi2 = 11.62, $P < 0.05$). Networks built separately for the two blocks thus defined make it easier to understand the relationships between haplotypes (Fig. 3B,C). The proximal segment shows the four mutations distinguishing *L. granatensis* haplogroups 1 and 2 and making the latter closer to *L. timidus*. We therefore make the hypothesis that in this proximal segment, haplotypes G1–G4 are of *granatensis* origin, and haplotypes G5–G9 of *timidus* origin. Understanding the complete history of these haplotypes depends on the

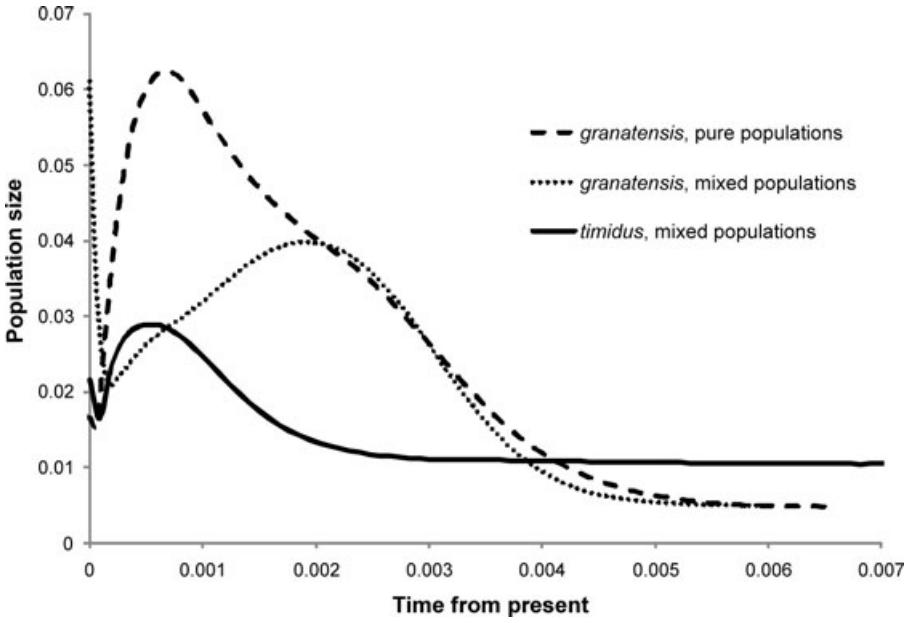

Figure 2. Skyline plots inferred for three different mtDNA datasets, as indicated in the legend. Values of time and population sizes on the axes are scaled with mutation rates. Applying a mutation rate of 7.9×10^{-02} substitutions/site×My (Melo-Ferreira et al. 2007), 0.0060 substitutions/site correspond to about 75,000 years BP.

determination of the origin of the distal segment. There is no diagnostic mutation between the G and T haplotypes in this segment. Let us first suppose that the G haplotypes are of *granatensis* origin in this distal segment, and there is just not enough resolution to fully distinguish the two species. We therefore have to reconstruct the recombination events by which haplotypes G5–G9 got their distal *timidus* segment. This must be through recombination in the distal vicinity of site 435, and obviously several recombinations in roughly the same position and involving different haplotypes are needed to account for the nucleotide diversities

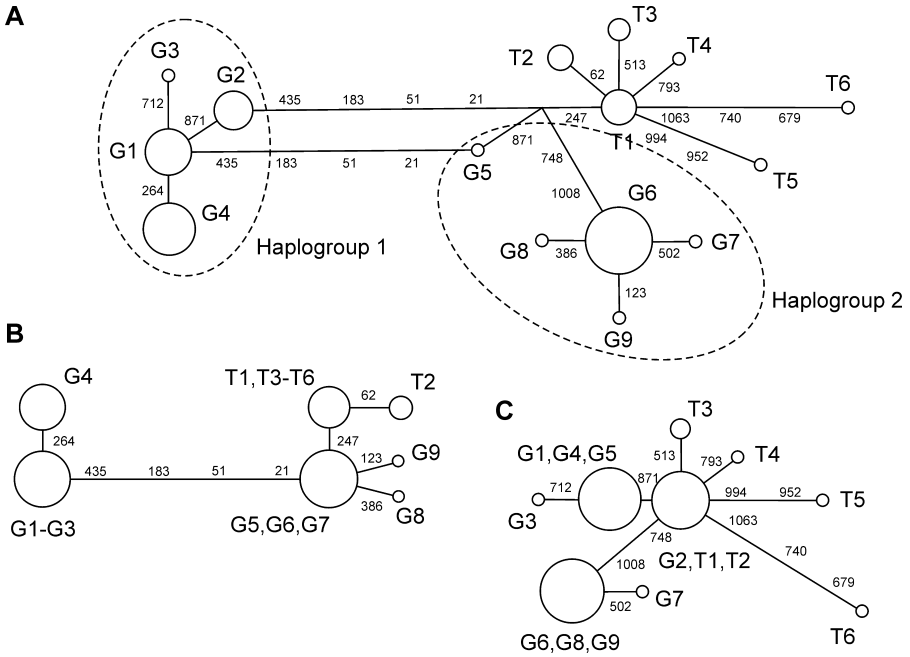

Figure 3. Median-joining haplotype networks constructed for PHKA2: (A) total fragment, (B) 5′ region, and (C) 3′ region. Circle sizes are proportional to haplotype frequencies. Each nucleotide substitution is identified along the branch where it occurs by its position in the alignment. Haplotypes found in *L. granatensis* and *L. timidus* are represented by letters G and T, respectively.

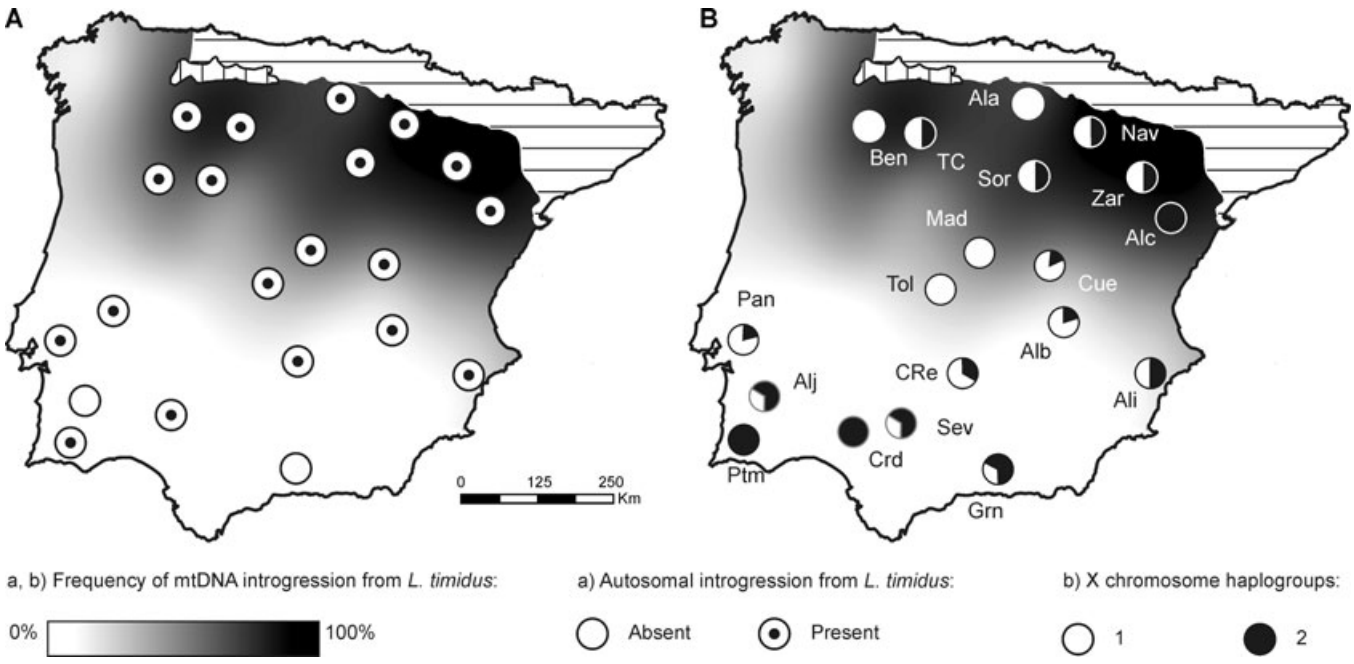

Figure 4. Geographic patterns of introgression from *L. timidus* into *L. granatensis*. mtDNA is represented by a color gradient on both maps, the darker the more introgression (data from Alves et al. 2008). (A) Localities with and without autosomal introgression (data from Melo-Ferreira et al. 2009), (B) populations analyzed for PHKA2 on the X chromosome (pie charts indicate the frequencies of the two haplogroups colored according to the legend). Striped areas show the distributions of two other species (horizontal, *L. europaeus*; vertical, *L. castroviejoi*).

found among these haplotypes both in the proximal and distal regions, although some of the derived mutations could have occurred after a recombination event. Another hypothesis is that in the distal segment, all haplotypes are of *timidus* origin. The distal network (Fig. 3C) would therefore attest of multiple introductions from *timidus* into *granatensis*. However, for the proximal region to regain the *L. granatensis* variation by recombination after introgression one must assume that "pure" *granatensis* haplotypes were still present in the population, which is not the case in our dataset. Whatever the interpretation, the high frequency of both haplogroups all over Iberia suggests extensive introgression, and even possible complete replacement in the distal segment.

The geographic distribution of PHKA2 haplotypes is given in detail in Table S4, and the map of Fig. 4B shows that in *L. granatensis*, both haplogroups 1 and 2 are found at high frequencies all over Iberia. The frequency of haplogroup 2 tends to increase from the centre of the Iberian Peninsula toward the periphery (correlation between frequency and distance to central population Toledo; $r^2 = 0.237$, $P = 0.034$).

Discussion

We have summarized in Figure 4 our present knowledge of introgression patterns from *L. timidus* into *L. granatensis* over its range for markers with different modes of transmission, to highlight the striking differences observed. Note that we did not report the data on two centromeric X markers and the Y, for which no introgression was detected (Melo-Ferreira et al. 2009). We first want to evaluate the compatibility of these patterns with the simple hypothesis that introgression was determined by the invasive replacement of *L. timidus* by *L. granatensis* in northern Iberia during the last deglaciation.

As noted in earlier studies, the limited geographical range of mtDNA introgression and its northward gradient of increasing frequency (Melo-Ferreira et al. 2005, pattern confirmed in the present study) can be expected if replacement with hybridization occurred during colonization by *L. granatensis* of northern Iberia then occupied by *L. timidus*. The increase in introgression frequency along the direction of colonization is a predictable consequence of surfing on the expansion wave, and of repeated introgression along the progression front (reviewed in Excoffier et al. 2009).

We found mtDNA of *L. timidus* origin to be highly phylogeographically structured, with three lineages almost completely geographically separated from east to west (Fig. 1A). Segregation of pre-existing variation in defined sectors of a newly colonized area has been observed in experiments with microorganisms and is a predictable consequence of drift at the early stages of colonization (Hallatschek et al. 2007; Hallatschek and Nelson 2010). Because in two dimensions initial sampling occurs along a direction perpendicular to the direction of expansion, differentiation along the direction of this initial sampling is expected to result

after expansion. However, interpreting the geographic separation of *timidus*-like mtDNA lineages in this way would imply that each of the three areas was colonized from a single foreign haplotype, and that all the diversity observed within each lineage was generated by mutation since these few initial introgressions. We attempted to test this by inferring the distribution of coalescence times in the mtDNA phylogeny and comparing it to the estimated date of expansion using the same method (which avoided the difficult problem of the calibration of the molecular clock). Although most coalescence events appeared younger than this expansion, it could not be excluded that a few be older (not shown), and thus a definite conclusion cannot be reached. Another observed pattern is notable in this context. Although the signal was only significant for one of the lineages, we found a northward increase of mtDNA variability. This would readily be explained if introgression events occurred all along the progression wave, thus gradually increasing variability northwards (Excoffier et al. 2009), but it is also possible that as the expansion front progressed, more derived mutations were available for surfing, thus contributing to increasing diversity. More sequencing data and some theoretical work appear needed before this issue can be properly addressed, but the question is rendered intrinsically difficult by the fact that the donor populations are extinct. Rejecting the possibility of a unique origin for each *timidus*-like lineage, we would have to suppose that the *L. timidus* population was geographically structured at the time of its replacement by *L. granatensis*. This might have corresponded to fragmentation of *L. timidus* into colder or higher altitude areas of the Iberian Peninsula. The present distribution of *L. timidus* is indeed fragmented in such a way, for instance between the Alps and Northern Europe. In addition, several phylogeography studies of Iberian taxa have shown that pockets of species persistence during the glacial periods are frequently associated with mountain chains (Gómez and Lunt 2007). Whichever hypothesis explains it, the strong phylogeographic structure of *L. timidus* mtDNA in Iberia attests that the process by which introgression occurred was repeated at least three times independently, leading each time to the same result, massive introgression. This illustrates that the phenomenon was quasi-deterministic, rather than a simple accident. However, models have shown that even if driven by purely stochastic processes, under certain conditions the outcome of introgression can be very probable during an invasion (Currat et al. 2008). Note that the east–west differentiation of introgressed lineages can also be taken as evidence that the wave of *L. granatensis* expansion was along a north–south axis. The contrasting absence of such structure in native *granatensis* haplotypes may be the witness of the population structure of Iberian *L. timidus* pre-existing introgression.

Our analysis of sequence variation of *timidus*-like mtDNA suggested an overall pattern of recent expansion, although the corresponding statistics were not always significant (*Fs* and *g* statistics in Table 2, skyline plot in Figs. 2 and S3). The date of this possible expansion of *timidus* mtDNA had been estimated on the basis of the mismatch distribution to be around 30,000 YBP (range around 20,000 to 80,000; Melo-Ferreira et al. 2007). Because this was based on calibrating the mutation rate from the evolution rate estimated at a broad evolutionary scale, this is likely to be an overestimate because of possible time dependency of the molecular clock (Ho et al. 2005; Henn et al. 2009). Although the extent of this time dependency and its origin are hotly debated issues (Woodhams 2006; Pulquerio and Nichols 2007; Navascués and Emerson 2009; Subramanian et al. 2009), at least one of the potential sources of dependency, that is, variations in mutation hot spots (Galtier et al. 2006; Pulquerio and Nichols 2007), is likely to apply on these CR sequences. Therefore although our rough estimate appears a bit too old, it may be overestimated and a postglacial expansion would be compatible with these results (see Melo-Ferreira et al. 2007), and in fact the skyline plot analysis appears more in line with the expectation of a postglacial expansion (Fig. 2). If such recent expansion of the *timidus*-like mtDNA lineage in Iberia resulted from the expansion of *L. granatensis* into the territory of *L. timidus*, one would expect to detect signs of a contemporaneous expansion among haplotypes of *granatensis* origin as well. The overall pattern we inferred from variation in the *granatensis* lineage points to a rather ancient demographic growth, largely predating that inferred from the *timidus* subset (Table 2). The palaeoclimatic conditions that could have favored such ancient growth are not clear because it would overlap at least one glacial cycle, but the skyline plot analysis (Fig. 2) suggests growth to have persisted during more recent periods coinciding with the demographic growth signal in the *timidus* lineage. An interesting point though is that such signal was not seen among *granatensis* haplotypes sampled from mixed populations where *timidus* haplotypes segregate. This pattern is in fact more compatible with a hypothesis of selective replacement of the *granatensis* mtDNA by *timidus* mtDNA in the north, with growth of the latter coinciding with shrinking of the former. Unfortunately the wide confidence intervals associated with these estimates of past demographic changes make these conclusions only tentative. We however find it striking that by analyzing the three independent datasets, we got such correlated responses. Being able to build and apply a population model to jointly analyze these three datasets would certainly give more power to the analysis, but an appropriate method is not available to our knowledge. We had also noted that, after taking into account large-scale geographic patterns, differentiation between populations appeared stronger for the *granatensis* haplogroup than for the *timidus* one in mixed populations (table 3). This observation would also better fit the selective than the demographic hypothesis to account for mtDNA introgression, but these comparison of Phi-statistics should be taken with caution (Meirmans and Hedrick 2011 for

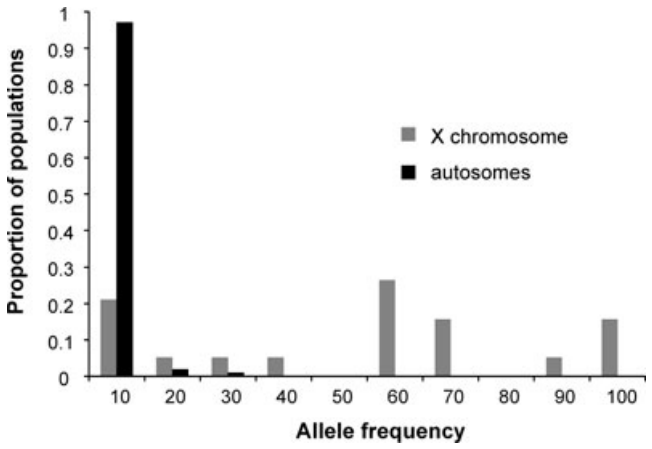

Figure 5. Distribution of the frequency of introgression of *L. timidus* origin across *L. granatensis* populations in the autosomes (data from Melo-Ferreira et al. 2009) and the X chromosome (haplogroup 2).

a recent review) because of differences in the levels of polymorphism between the two lineages (Table 2, although haplotype diversities are similar between the two lineages, nucleotide diversities are higher in the *granatensis* lineage than in the *timidus* sublineage).

To summarize our analyses of mtDNA variation, we found that many of the observed patterns are compatible with the predictions of the invasion-replacement hypothesis. However we also note that none of them is in contradiction with the selective hypothesis and that some observations would better fit the latter, but would need confirmation. We will now compare the results of mtDNA with those of the nuclear genome. Autosomal introgression contrasts with that of mtDNA, because it is only sporadic and not geographically limited (Fig. 4A). Some possible causes have been extensively discussed elsewhere (Melo-Ferreira et al. 2009), but under the invasion-replacement hypothesis, the lower level of introgression could be explained theoretically by higher male migration, although little is known of this aspect of *L. granatensis* ecology. The extension of introgression to the whole species range could also be a consequence of male-mediated migration occurring after introgression occurred in the north.

The extensiveness of the observed introgression at the PHKA2 X-linked locus appears at odds with the patterns for mtDNA and the autosomes. Under one of the possible interpretations of variation at PHKA2, a distal portion of the fragment of *L. timidus* origin is fixed in *L. granatensis*, and the proximal portion is highly and ubiquitously introgressed. Under the alternative interpretation, only haplogroup 2 has *timidus* ancestry (in its distal part) but introgression is still pervasive (Fig. 4B). In Figure 5 we compare the distribution of introgression frequencies among populations between the autosomal loci and PHKA2, showing clearly greater introgression of this X locus, even if conservatively considering that only haplogroup 2 has *L. timidus* ancestry. This pervasive introgression of PHKA2 contrasts with the absence of introgression reported for two centromeric X markers, with the rarity of introgression at 10 autosomal loci and also with its restriction to the north for mtDNA. The level of interspecific introgression of nuclear genes can depend on their degree of linkage to hybrid incompatibility factors. The X chromosome is known to frequently harbor such factors (Hemberger et al. 1999; Storchova et al. 2004; Britton-Davidian et al. 2005; Oka et al. 2007; Good et al. 2008; Presgraves 2008), and the centromeric region is typically low recombining, which could account for the absence of introgression of this genomic region. The PHKA2 gene is telomeric on the rabbit X and presumably so also in hares given the high level of synteny between the two genomes (Robinson et al. 2002). Because recombination is typically high in telomeric regions, this could account for easier introgression than for centromeric markers, as documented for instance in rabbits (Carneiro et al. 2009; but see Carneiro et al. 2010), but the difference with the autosomes would suggest that the 10 autosomal loci studied by Melo-Ferreira et al. (2009) are closely linked to incompatibility factors. Even if such was the case, the extent of *timidus* PHKA2 introgression would be striking. It is conceivable that this happened by chance in the north under the hypothesis of invasive replacement, because drift would have been important during the invasion. But extension to the whole species including in established populations appears very unlikely solely by drift and raises the strong possibility that PHKA2 introgression was favored by selection. In fact it is not too hard to imagine an X factor conferring an advantage specifically to females (Gibson et al. 2002; Balaresque et al. 2004).

Alternatively, the pattern of species-wide introgression of nuclear markers might reflect a colonization pattern different from the one elaborated on the basis of the geographically restricted mtDNA introgression. One could suppose that *L. granatensis* acquired its present pan-Iberian distribution by expansion from a refugium in central Spain. Even if initially rare, traces of hybridization events with *L. timidus* occurring at that time and there (presumably in the southernmost range of *L. timidus*) could have been propagated during the expansion of *L. granatensis*, at least for some nuclear fragments, thus accounting for their broad present distribution. Note that this is not incompatible with the supposed replacement of *L. timidus* by *L. granatensis* in the north, accounting for the massive mtDNA introgression only there. This would point to central Iberia as a refuge zone for *L. granatensis*. The Iberian Peninsula has a complex physiography, with several mountain ranges and rivers and presents several different climatic regions. Therefore, the fragmentation and/or restriction of suitable habitats for each organism, coupled with the historical population progression contributed to restrict the distribution of taxa at the glacial maxima in a generally idiosyncratic manner (see Weiss

and Ferrand 2007 for a general appreciation). It is possible that *L. granatensis* restricted its past distribution to the area between the Spanish Central System and the Betic System mountains and the existence of two refuges could even have helped retain the two divergent lineages now surviving in different geographic areas (Fig. 1B). Phylogeographic studies of Iberian taxa have suggested that the Iberian Peninsula was not a homogeneous deposit of genetic diversity. Instead, organisms in this area, such as plants (e.g., Petit et al. 2002), reptiles (Pinho et al. 2007; Godinho et al. 2008), amphibians (Alexandrino et al. 2000; Goncalves et al. 2009), or mammals (Branco et al. 2002) show remarkably complex patterns of fragmentation of genetic diversity, suggesting the existence of refugia within the major refugium (reviewed in Gómez and Lunt 2007).

Yet, the pervasiveness and high frequency of PHKA2 introgression is striking, and it could be that it was favored precisely under such a situation of geographic expansion, which might account for its observed increased frequency from central Iberia to its more recently colonized periphery. In situations of colonization, female number is a limiting factor to demographic expansion, and female-biased sex-ratios could be favored. X-borne factors biasing the sex-ratio in favor of females have been described, acting either as transmission distorters (Montchamp-Moreau and Cazemajor 2002; Montchamp-Moreau 2006; Tao et al. 2007) or as feminizing factors (Givela 1987; Bulmer 1988; Liu et al. 1998; Veyrunes et al. 2010). The possibility that the introgressed X-chromosome fragment be linked to a sex-ratio distorter relates to a controversial issue. Genetic conflicts such as those over sex-ratio control have been suggested as potential factors promoting speciation, because by essence they imply rapid coevolution between genes with conflicting interests (Frank 1991; Tao et al. 2001; Orr and Irving 2005; Presgraves 2008; Phadnis and Orr 2009). If it turned out to have sex-ratio distortion properties, the invasive X fragment in hares would on the contrary document a case of genetic introgression promoted by the consequences of such a genetic conflict. Interestingly, a recent report (Macholan et al. 2008) described a local Y chromosome incursion across the European house mouse hybrid zone (elsewhere perfectly impermeable to introgression of this chromosome), coinciding with a local change in population sex-ratio. Thus although this possibility is purely speculative, we feel it would deserve further consideration in hares.

In conclusion, we have seen through this case study on hares how contrasted interspecific introgression patterns can be between genomic regions. Because the effects of demography and selection can be so similar, a precise and independent knowledge of colonization and demographic history is needed because inference of history and selection from the same dataset potentially leads to circular reasoning. Data on many loci and specific modeling of colonization scenarios will be needed to make progress. Hares promise to be a rich model for comparative studies of reticulation because extensive mtDNA introgression has been documented in two other species in the Iberian Peninsula (*L. europaeus* and *L. castroviejoi*, Melo-Ferreira et al. 2005; Alves et al. 2008; Melo-Ferreira et al. 2009) and is suspected in several other cases in Asia and America (Alves et al. 2006; Alves et al. 2008).

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Supporting Information

The following supporting information is available for this article:

Figure S1. Median-joining networks of mtDNA haplotypes in *L. granatensis*, of either *timidus* (A) or *granatensis* (B) origin.
Figure S2. mtDNA pairwise mismatch distributions for (A) the *granatensis*-like and (B) the *timidus*-like mtDNA lineages.
Figure S3. 95% confidence intervals for the Bayesian Skyline plots of Fig. 2.
Table S1. Geographic coordinates of population samples and frequencies of *timidus* mtDNA (from Alves et al. 2008b).
Table S2. Genetic diversity indices for mtDNA of *granatensis* and *timidus* origins per sampled locality.
Table S3. In each locality, the mitochondrial DNA haplotypes detected, named according to Fig. S1 and preceeded by their frequency.
Table S4. Frequencies of the PHKA2 sequence haplotypes in *L. granatensis* (*gra*) and *L. timidus* (*tim*) samples.

Supporting Information may be found in the online version of this article.

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