Heredity (2015) 115, 444–451
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www.nature.com/hdy

ORIGINAL ARTICLE

p53 gene discriminates two ecologically divergent sister species of pine voles

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Genes with relevant roles in the differentiation of closely-related species are likely to have diverged simultaneously with the species and more accurately reproduce the species tree. The Lusitanian (*Microtus lusitanicus*) and Mediterranean (*M. duodecimcostatus*) pine voles are two recently separated sister species with fossorial lifestyles whose different ecological, physiological and morphological phenotypes reflect the better adaptation of *M. duodecimcostatus* to the underground habitat. Here we asked whether the differentiation of *M. lusitanicus* and *M. duodecimcostatus* involved genetic variations within the tumour suppressor *p53* gene, given its role in stress-associated responses. We performed a population-genetic analysis through sequencing of exons and introns of *p53* in individuals from sympatric and allopatric populations of both the species in the Iberian Peninsula in which a unidirectional introgression of mitochondrial DNA was previously observed. We were able to discriminate the two species to a large extent. We show that *M. duodecimcostatus* is composed of one genetically unstructured group of populations sharing a P53 protein that carries a mutation in the DNA-binding region not observed in *M. lusitanicus*, raising the possibility that this mutation may have been central in the evolutionary history of *M. duodecimcostatus*. Our results provide suggestive evidence for the involvement of a master transcription factor in the separation of *M. lusitanicus* and *M. duodecimcostatus* during *Microtus* radiation in the Quaternary presumably via a differential adaptive role of the novel *p53* in *M. duodecimcostatus*.
Heredity (2015) **115,** 444–451; doi:10.1038/hdy.2015.44; published online 20 May 2015

INTRODUCTION

Microtus lusitanicus and *M. duodecimcostatus* (the Lusitanian and the Mediterranean pine voles, respectively) are western European endemic sister species, with an estimated origin of <150 Kyr (Brunet-Lecomte and Chaline, 1991; Tougard *et al.*, 2008). Both the species exhibit burrowing behaviour but differ in a set of ecological and morphological traits that together suggest that *M. duodecimcostatus* is more dependent from the subterranean habitat than *M. lusitanicus* (Mathias, 1990; Santos *et al.*, 2009a,b; Santos *et al.*, 2010). Living underground forces rebreathe of air with low oxygen (hypoxia) and high carbon dioxide tensions (hypercapnia). Respiratory properties and acid–base parameters in the blood of *M. duodecimcostatus* suggest that this species has developed features to adequately deliver oxygen to tissues under hypoxic-hypercapnic stress (Mathias and Freitas, 1989). Similarly, subterranean species of the genus *Spalax* (blind mole rat) have evolved physiological and genetic adaptations to efficiently cope with hypoxia (Nevo, 2011). One such genetic variation is located within the tumour suppressor *p53* gene (Ashur-Fabian *et al.*, 2004), the most mutated gene in human cancers (Vogelstein *et al.*, 2000). This gene encodes an important transcription factor with constitutive functions in cell cycle arrest, DNA repair, senescence, apoptosis and metabolism in response to stimuli like hypoxia, nutrient deprivation, genotoxic and oxidative stress (Brady and Attardi, 2010). The *p53* mutation found in *Spalax*—R174K in the human protein—was shown to be sufficient to affect the transcriptional activity of both *Spalax* and human P53 protein (Ashur-Fabian *et al.*, 2004; Avivi *et al.*, 2007). Other codon variations in P53 were shown to provide adaptive responses to a wide range of environmental cues in other species (Villiard *et al.*, 2007; Zhao *et al.*, 2013), strengthening the hypothesis that P53 protein may adapt in structure and function in accordance with ecological stress (Zhao *et al.*, 2013).

Under this assumption and considering the underground dependence of *M. lusitanicus* and *M. duodecimcostatus*, we decided to investigate the presence of genetic variations in the *p53* gene in both the species. The two existing population-level studies on the genetic diversity of *M. lusitanicus* and *M. duodecimcostatus* in the Iberian Peninsula have found evidence for a past introgressive hybridization from *M. duodecimcustatus* to *M. lusitanicus* of the mitochondrial cytochrome b (*cytb*) gene (Bastos-Silveira *et al.*, 2012), and possibly of the nuclear gene *IRBP* (interphotoreceptor retinoid-binding protein) given the impossibility to discriminate individuals from the two species when using this marker (Barbosa *et al.*, 2013). Here we performed a population-genetic analysis involving 96 individuals (52 *M. lusitanicus* and 44 *M. duodecimcostatus*) sampled in the Iberian

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Received 21 November 2014; revised 10 March 2015; accepted 24 March 2015; published online 20 May 2015

Peninsula, and the *p53* DNA sequence encoding part of the DNA-binding region of P53, from the 3′ end of exon 5 to the 5′ extreme of exon 7, which includes residue 174. We used specimens captured both in allopatric and sympatric areas where introgressed animals have been observed (Jaarola *et al.*, 2004; Tougard *et al.*, 2008; Bastos-Silveira *et al.*, 2012; Barbosa *et al.*, 2013; Rodriguez-Prieto *et al.*, 2014), to ultimately evaluate whether genes involved in the differentiation of species separated recently carry signature changes reflecting speciation more accurately than neutral genes (Ting *et al.*, 2000). Variability of the obtained *p53*-coding sequences of *M. lusitanicus* and *M. duodecimcostatus* was further compared with the published sequences from other *Microtus* species, rodents and mammals.

MATERIALS AND METHODS

Samples

M. lusitanicus and *M. duodecimcostatus* specimens were collected from 21 different sampling sites, throughout the species distribution range in the Iberian Peninsula (Supplementary Table S1). Thirty-one of these individuals had also been studied for *cytb* sequence diversity in a previous study (Bastos-Silveira *et al.*, 2012). Ten samples from *M. duodecimcostatus* (from sampling sites 21, 22 and 23) came from the Museo Nacional de Ciencias Naturales, Spain, as well as one sample from *M. cabrerae* that was used for sequence comparisons within *Microtus*.

DNA extraction, *p53* amplification and sequencing

Total genomic DNA was extracted from the liver or tail fragments conserved in ethanol using a standard phenol–chloroform protocol. Amplification conditions were as in (DeWoody, 1999), with primer pair p53C and p53D used for amplification and sequencing reactions. PCR products were sequenced on both the strands by Macrogen Inc (Seoul, Korea).

p53 sequence analyses

p53 sequences were aligned using the Clustal W algorithm (Thompson *et al.*, 1994), revised and edited manually in BioEdit 7.2.3 (Hall, 1999). Repeat motifs of polymorphic insertion/deletions (indels) found in intron 6 of *p53* were kept clustered. *p53* alleles from heterozygous individuals were inferred using PHASE within DnaSP 5.10.01 (Librado and Rozas, 2009). Five replicate PHASE runs were conducted using default values before a final run with 100 burn-in steps and 1000 iterations. The PHASE probability threshold was set to 0.90. Shared sequence types were collapsed into haplotypes using the program DNAcollapser (Villesen, 2007).

Exonic sequences were translated into amino acid (AA) sequences in Mega 5.2.2 (Tamura *et al.*, 2011) using the universal code. Positions of AA were determined using the structured model of human P53. Known sequences of the *p53* gene from *M. agrestis*, *M. arvalis*, *M. oeconomus*, *M. rossiaemeridionalis* and *M. ochrogaster* were obtained in GenBank (accession numbers listed in Supplementary Table S2), and used for sequence comparisons within *Microtus*.

Phylogenetic tree and haplotype network construction for *p53* and *cytb*

Phylogenetic relationships among haplotypes were reconstructed by either maximum likelihood in Mega 5.2.2, or using Bayesian inference as implemented in MrBayes 3.2.2 (Ronquist *et al.*, 2012). The program JModelTest 2.1.4 (Darriba *et al.*, 2012) was used to estimate the best DNA substitution model for a set of sequences by performing hierarchical likelihood ratio tests to compare 88 different models and applying the Akaike Information Criterion under default settings. For *p53* haplotypes, the TPM2uf model was chosen with uniform rates of variable sites with the following estimated nucleotide frequencies: A = 0.1947, C = 0.2537, G = 0.2938 and T = 0.2578; and substitution rate matrix: (AC) = 1495.6642, (AG) = 8493.0230, (AT) = 1495.6642, (CG) = 1.0000, (CT) = 8492.0230 and (GT) = 1.0000. Because a regional partition of *cytb* haplotypes according to introgression status was observed in *M. lusitanicus* (Bastos-Silveira *et al.*, 2012; Barbosa *et al.*, 2013), we reconstructed the phylogenetic tree of *M. lusitanicus* and *M. duodecimcostatus* using all the *cytb* sequences available in GenBank and assigned each specimen to its place of origin to investigate any connection between the geographical distribution of *p53* and *cytb* haplotypes. A total of 129 *cytb* haplotypes were retrieved from all the published sequences of each species, 77 haplotypes from 79 individuals of *M. lusitanicus* and 52 from 55 specimens of *M. duodecimcostatus* (accession numbers in Supplementary Table S2; Jaarola *et al.*, 2004; Tougard *et al.*, 2008; Bastos-Silveira *et al.*, 2012; Barbosa *et al.*, 2013). For the construction of the *cytb* phylogenetic tree the TIM3 substitution model was chosen with a proportion of invariable sites and a gamma-distributed rate variation across sites (TIM3+I+G). The estimated parameters of the model were as follows: substitution rate matrix (AC) = 2.1238, (AG) = 40.0258, (AT) = 1.0000, (CG) = 2.1238, (CT) = 15.2400 and (GT) = 1.0000; gamma shape parameter = 0.8630, with 70.9% of invariable sites; and nucleotide frequencies of A = 0.3117, C = 0.3030, G = 0.1307 and T = 0.2547. Bayesian inference analysis was performed with two independent runs with four chains (one cold and three hot chains) for one (*p53*) or ten (*cytb*) million generations, with a sample frequency of 100. Average s.d. of the split frequencies between the independent runs was checked for convergence on a stationary distribution (Ronquist *et al.*, 2012). The first 25% of the trees were discarded as burn-in and the remaining trees were used to reconstruct a consensus tree and estimate Bayesian posterior probabilities. Maximum likelihood trees were constructed for *p53* haplotypes with 10 000 bootstrap replicates. Gene topologies for *p53* were inferred using the median-joining network construction approach as computed with Network 4.6.1.2 (Bandelt *et al.*, 1999). Phylogenetic and network analyses were rooted using gene sequences of *M. rossiaemeridionalis* (= *M. levis*; GenBank accession number AF014024) or of *M. gerbei* (GenBank accession numbers in Supplementary Table S2).

Genetic diversity and demographic analyses on *p53* sequences

DnaSP 5.10.01 was used to determine nucleotide diversity (π), haplotype number (h) and diversity (Hd), and number of variable sites. Genetic differentiation among the groups of samples of *M. lusitanicus* and *M.duodecimcostatus* was assessed by the analysis of molecular variance of the *p53* sequences using Arlequin 3.5.1.2 (Excoffier and Lischer, 2010). Signals of departure from neutrality were tested using Tajima's D and Fu's Fs statistics. Statistics based on the mismatch distribution were used to test for demographic expansions under a sudden or a spatial model. Extra repeat motifs found in intron 6 (indel of 10 bp) were removed to allow pairwise comparisons between all the sequences. Statistically significant differences between the observed and the simulated expected mismatch distributions (1000 bootstraps) were evaluated with the sum of the square deviations and Harpending's raggedness index (hg), using Arlequin 3.5.1.2. Frequency distribution graphs were obtained using DNAsp 5.10.01.

Selection tests on *p53* exonic sequences under different codon substitution models

Using the *Microtus* genus phylogenetic tree obtained with exonic nucleotide sequences of the *p53* fragment (189 bp), the rate of non-synonymous and synonymous substitutions (ω) of each branch was calculated with PAML 4.7 (Yang, 2007). In addition, each codon of the 63aa P53 fragment was tested for selection pressure by site-specific models. To contrast *Microtus p53* diversity with variability within rodents and mammals, we supplemented our data with available nucleotide sequences of this gene region and constructed rodent and mammalian *p53*-based phylogenies. Unrooted phylogenetic trees using GenBank retrieved *p53* exonic sequences covering the 3′ end of exon 5 to the 5′ extreme of exon 7 (189 bp) of *Microtus* (8 species), rodents (21 species) and mammals (60 species) were obtained with MrBayes 3.2.2 and used as input data in PAML 4.7. Species used in these analyses and respective *p53* accession numbers are listed in Supplementary Tables S2 and S3. Bayesian inference analysis was performed as before. JModelTest 2.1.4 selected the TPM2 model as the best substitution model for the *Microtus* phylogeny (substitution rate matrix: (AC) = 1638.9541, (AG) = 3746.4665, (AT) = 1638.9541, (CG) = 1.0000, (CT) = 3746.4665 and (GT) = 1.0000), the TPM2uf+I+G model for the rodent phylogeny (with gamma shape parameter = 2.5410, 52.7% of invariable sites, nucleotide frequencies of A = 0.1970, C = 0.3098, G = 0.2908 and T = 0.2024; substitution rate matrix: (AC) = 2.7868, (AG) = 13.8676, (AT) = 2.7868, (CG) = 1.0000, (CT) = 13.8676 and (GT) = 1.0000), and the

HKY+I+G model for the mammalian phylogeny (gamma shape parameter = 0.8430, with 30.6% of invariable sites; and nucleotide frequencies of A = 0.2267, C = 0.3287, G = 0.2467 and T = 0.1979). Statistical tests for selection were done using codon-based maximum likelihood methods implemented in the program 'codeml' from PAML 4.7. Five comparisons of paired models, M0-model = 2, M1–M2, M0–M3, M7–M8 and the branch-site test of positive selection, were used for the likelihood ratio test. The M0-model = 2 comparison is suitable to test if the ω ratio (d*N*/d*S* ratio) for a particular branch is different from that for all other branches. Likelihood ratio tests of M1–M2 and M7–M8 are useful to examine positive selection acting on codons. The M0–M3 comparison tests the variable pressure among sites. Branch-site test of positive selection compares the modified model A with the corresponding null model with $\omega_2 = 1$ fixed and aims to detect positive selection on sites along the foreground branch. Posterior probabilities for positively selected sites were calculated with Naïve Empirical Bayes for model M3 and Bayes Empirical Bayes for models M2, M8 and model A. Model A was replicated with different starting omega values to ensure convergence of the likelihood. Gaps in the alignment were treated as ambiguity characters in likelihood calculations.

RESULTS

p53 DNA sequence diversity

The amplified sequence (697 bp) revealed that a tandem repeat of 20 bp ((GGTTT)$_3$GATTT) within intron 6, common to other *Microtus* species (DeWoody, 1999), contained one or two extra repeat motifs of 5 bp (GGTTT) in at least one *p53* allele in 29 individuals of *M. lusitanicus*. We found seven single-nucleotide polymorphisms in *M. lusitanicus* and nine in *M. duodecimcostatus* (Table 1), of which three and eight, respectively, were parsimony informative. The seven single-nucleotide polymorphisms found in *M. lusitanicus* were all located in intron 6, whereas in *M. duodecimcostatus* the variable sites were scattered along the introns and exons of the analysed sequence. A total of 21 haplotypes were found, 12 in *M. lusitanicus* and 10 in *M. duodecimcostatus* (Figure 1a, Table 1 and Supplementary Table S1).

Phylogenetic and network relationships among *p53* haplotypes

The phylogenetic tree of *p53* was comprised by one lineage with high posterior probability corresponding to *M. duodecimcostatus* (Figure 1b). The most common haplotype in *M. lusitanicus* (haplotype 5) was shared with *M. duodecimcostatus* (54 and 18% of samples, respectively). Haplotypes 8, 9, 13, 14 and 17 to 21 were exclusive of *M. lusitanicus* sampled to the north of the Douro river, while *M. lusitanicus* located southward of this river only carried haplotypes 5–7 (Figures 1a and b). The network displayed three unresolved reticulations because of an indel in intron 6 (one or two extra repeat motifs of 5 bp) (Figure 1c). Ninety-seven percent of *M. lusitanicus* from the northern region carried this indel, 65% of them in both the alleles. One mutation in intron 5 separated the two main genetic groups, between haplotypes 5 and 12, the latter appearing exclusively in *M. duodecimcostatus*. The most frequent haplotype in *M. duodecimcostatus* was haplotype 1 (82% of samples), which connected to haplotype 12 by one mutational step that corresponded to a non-synonymous mutation in exon 7. The root of the network was located along the branch that connects haplotype 5 to *M. rossiaemeridionalis* haplotype (outgroup), suggesting that haplotype 5 may be the ancestral haplotype between *M. lusitanicus* and *M. duodecimcostatus*.

Table 1 Sequence diversity values, fixation indices and sources of variation in *p53* sequences from *M. lusitanicus* and *M. duodecimcostatus*

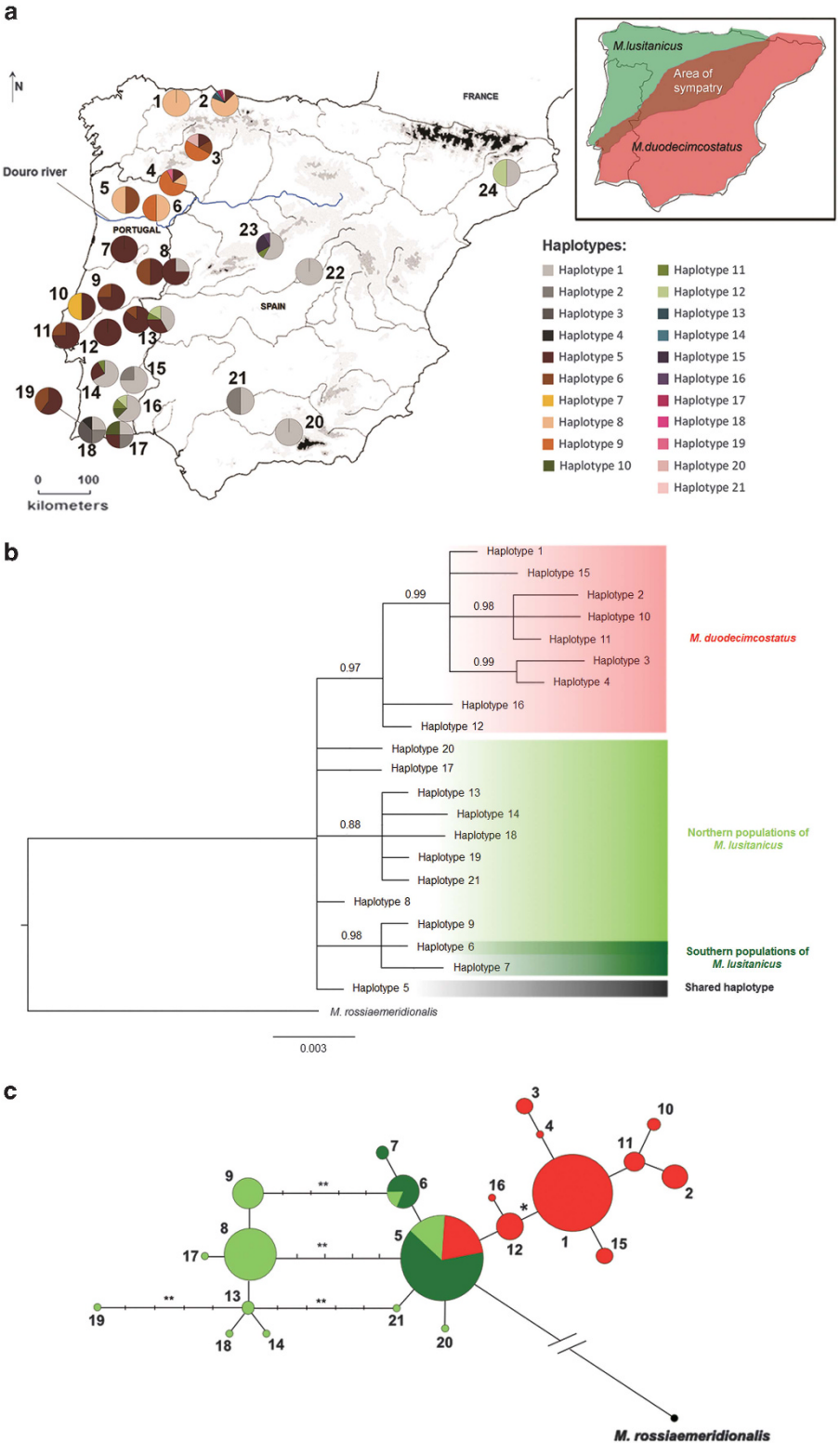
Species (region)	*Number of sequences*	*Sequence diversity*				*Fixation indices and source of variation*				
		Number of segregating sites[a]	$\pi \pm$ s.d.[b]	*h*[b]	*Hd* ± s.d.[b]	ϕ_{ST}	ϕ_{CT}	ϕ_{SC}	*% Among groups*	*% Within populations*
Microtus lusitanicus	104	7	0.0046 ± 0.0027	12	0.748 ± 0.026					
(All populations)						0.576				42.37
(Northern group)/(southern group)						0.697	0.670	0.080	67.04	30.32
(Northern group)	60	6	0.0034 ± 0.0021	11	0.697 ± 0.051	0.129				87.09
(Southern group)	44	2	0.0007 ± 0.0001	3	0.403 ± 0.076	0.055				94.51
Microtus duodecimcostatus	88	9	0.0017 ± 0.0002	10	0.629 ± 0.055					
(All populations)						0.190				81.04

[a]indel of 10 bp not included.
[b]π, nucleotide diversity; h, number of haplotypes; Hd,haplotype diversity.

Figure 1 Distribution of *p53* haplotypes in the Iberian Peninsula and respective phylogenetic and network relationships. (**a**) Map of the Iberian Peninsula showing frequency distributions of *p53* haplotypes from *M. lusitanicus* and *M. duodecimcostatus* according to sampling site (numbered 1 to 24). In sampling sites 8 and 13 the left pie corresponds to *M. lusitanicus* and the right pie to *M. duodecimcostatus*. Sampling sites and haplotype labels are as in Supplementary Table S1. Shaded areas of the map denote altitude gradients, with darker areas corresponding to higher altitudes. Douro river is highlighted in blue. Top right corner: distribution range of each species in the Iberian Peninsula (green: *M. lusitanicus*; red: *M. duodecimcostatus*; brown: area of sympatry). (**b**) Phylogenetic relationships among *p53* haplotypes, according to the Bayesian inference analysis and with *M. rossiaemeridionalis* as the outgroup. Numbers above branches represent posterior probabilities. Maximum likelihood inferred the same topology but with lower branch support. Northern and southern populations of *M. lusitanicus* are located north- and southward of the Douro river, respectively. (**c**) Median-joining network of the 21 *p53* haplotypes found in *M. lusitanicus* and *M. duodecimcostatus* and the haplotype from *M. rossiaemeridionalis*. Numbers of circles correspond to the respective haplotypes as in **a** and **b**. Colours of circles correspond to species and regional populations as in **b**. Areas of the circles in the network are proportional to the haplotype frequency. Each bar on the connecting lines separates two mutational events except between haplotype 5 and haplotype from *M. rossiaemeridionalis* (18 mutational steps). * Mutational event corresponding to a non-synonymous mutation in P53 protein; ** Mutational event corresponding to an additional repeat motif (5 bp) in intron 6.

Geographical distribution of *p53* sequence diversity

The analysis of molecular variance revealed no genetic structure for *M. duodecimcostatus*, indicating that the global source of variation is within and not among groups (Table 1). A genetic structuring was found for *M. lusitanicus* when sampling sites were divided in two regional groups, one comprised by the localities north of the Douro river and another by the localities south of this river, indicating that the overall source of variation is among the regions (67.04%). Overall, the southern group of *M. lusitanicus* showed lower nucleotide diversity and ϕ_{ST} value than *M. duodecimcostatus* and the northern group of *M. lusitanicus* (Table 1). The separation in two regional groups of populations for *M. lusitanicus* is also apparent in the reconstructed



cytb phylogenetic gene tree (Figure 2). Two lineages with high posterior probabilities were observed, one that corresponded to *M. lusitanicus* of the northern regional group and the other to a cluster comprised of *M. duodecimcostatus* and introgressed individuals of *M. lusitanicus* (Bastos-Silveira *et al.*, 2012; Barbosa *et al.*, 2013). Notably, these introgressed *M. lusitanicus* were also subdivided with high posterior probability in the two regional groups separated by the Douro river, suggesting that regional separation occurred after secondary contact between *M. lusitanicus* and *M. duodecimcostatus*.

Mismatch distribution analyses using *p53* sequences suggested past population range expansion (sudden-expansion and spatial-expansion models) for *M. duodecimcostatus* and the southern group of *M. lusitanicus*, while for the northern group of *M. lusitanicus* the observed distributions were significantly different from that expected under a spatial-expansion model according to sum of the square deviation (Supplementary Figure S1 and Supplementary Table S4).

P53 protein diversity

The 697-bp amplified fragment of *p53* encodes a region of 63 AAs. We found two types of coded sequences, one in *M. duodecimcostatus* and one in other *Microtus* species (Figure 3). The R174K mutation was not present in *Microtus*. However, *M. duodecimcostatus* carried a unique non-synonymous mutation at position 694, in exon 7. This mutation was a C–A transversion in the third position of codon 228 (residue number in the human protein), resulting in a conservative AA replacement of an aspartic acid (D) for a glutamic acid (E). It was found in all but four individuals (91% of the total sample) that came from the sympatric area of the distribution range. Two individuals from sampling sites 16 and 17 (allopatry; Figure 1a) carried an additional synonymous mutation at site 186, in exon 6 (C–A transversion in one *p53* allele; R202R; *M. duodecimcostatus2* in Figure 3a).

Phylogenetic analysis by maximum likelihood of *p53*-coding sequence

All comparisons of M0 with branch model = 2 suggested a significant different ω ratio for the *M. duodecimcostatus* branch, a result not observed when the *M. lusitanicus* branch is chosen (Supplementary Table S5). Site-selection tests also suggested a general negative selection acting on *p53* codons (M0, $\omega = 0.07$, $\omega = 0.07$ and $\omega = 0.08$ among *Microtus,* rodents and mammals, respectively), but with significant variation among sites in rodents and mammals (M0–M3, $P < 0.01$), implying that the selective pressure on this region varies among AAs. Model M3 indicated for the mammalian lineages only site 209 with high Naïve Empirical Bayes posterior probability for being positively selected. Branch-site tests of positive selection with *M. duodecimcostatus* as the foreground branch were not significant in the three sets of lineages analysed, although in all the cases branch-site model A predicted site 228 as potentially being under positive selection in this branch when compared with the background branches (Bayes Empirical Bayes posterior probabilities > 0.94). When selecting *M. lusitanicus* as the foreground branch, branch-site model A returned much lower posterior probabilities that this site is under positive selection in this branch (posterior probabilities < 0.32).

DISCUSSION

We did not find the R174K variation of P53 in either *M. lusitanicus* or *M. duodecimcostatus*. We were particularly interested in the latter because of its more pronounced underground habits. This mutation, although associated with hypoxic resistance in *Spalax*, is also not found in other subterranean taxa such as the naked mole rat (*Heterocephalus glaber*; Figure 3b), and may not be required *per se* to live in a hypoxic environment. Our most remarkable result, however, was the finding of a D228E mutation within the DNA-binding region of P53 in the majority of *M. duodecimcostatus*—91% of the total sample– and its absence in the other *Microtus* species analysed (*M. lusitanicus*, *M. agrestis*, *M. arvalis*, *M. oeconomus*, *M. rossiaemeridionalis, M. cabrerae* and *M. ochrogaster)*. This mutation could have appeared *de novo* in *M. duodecimcostatus* or existed as standing variation in ancestral populations, in which case we should have found it within *M. lusitanicus*, even at a low frequency. The absence of this alteration in other *Microtus* and the high frequency with which it is found in *M. duodecimcostatus* (68% in homozygosity) strongly suggests that this mutation may have had an important role in the differentiation of this species during the Pleistocene, when arvicolines (voles) in the Iberian Peninsula responded to the new environmental conditions with radiation to many ecological specialist species (Gomez Cano *et al.*, 2013). The largely allopatric distribution range of these two species may well reflect this ecological divergence. In sympatry, both the voles occupy a specialized ecological niche, in which soil characteristics seem to be particularly relevant (Borghi *et al.*, 1994; Santos *et al.*, 2009a, b; Santos *et al.*, 2010; Santos *et al.*, 2011). Niche specialization may involve distinctive physiological and genetic adaptations (Singh *et al.*, 2009; Hadid *et al.*, 2013), probably species and stress environmental specific, as suggested with the S104E variation observed within the P53 protein of high-altitude *M. oeconomus* or the R174K mutation in P53 of the subterranean *Spalax* (Ashur-Fabian *et al.*, 2004; Zhao *et al.*, 2013). The D228E mutation could similarly have favoured *M. duodecimcostatus* as this species expanded its territory in the Iberian Peninsula and evolved a more pronounced fossorial behaviour than *M. lusitanicus*.

Our phylogenetic analysis by maximum likelihood results are in agreement with analyses on the complete gene sequence that show the presence of mostly purifying sites in the DNA-binding domain of *p53* in mammals (Khan *et al.*, 2011). The non-significant results from the tests for positive selection most probably result from the fact that our analysed fragment is short-sized (63 codons) and shows low divergence among sequences, even when sampling is increased to include evolutionary distant mammalian taxa, resulting in low power of the likelihood ratio tests (Yang and Nielsen, 2002). It is worth noting, however, that parameter estimates under branch-site model A suggest the presence of sites under positive selection, namely site 228 for *M. duodecimcostatus*, with a posterior probability of $> 94\%$. The same test also predicts the physiologically important site 174 as being positively selected in the *Spalax* branch, with a posterior probability of $> 56\%$ (Supplementary Table S5). Lack of statistical support from the branch-site test of positive selection prevents us from interpreting these results as a conclusive evidence of positive selection acting on these sites (Zhang *et al.*, 2005), but clearly opens up the possibility that 228E may have a role in *M. duodecimcostatus* physiology, as already demonstrated for site 174K in *Spalax*, and that this issue should be addressed with functional studies.

As to the *p53* genetic structuring of *M. lusitanicus* and *M. duodecimcostatus* in the Iberian Peninsula, we found that *M. duodecimcostatus* is comprised of one genetically unstructured group of populations that possibly resulted from the range expansion of an ancestral population that carried haplotype 1 of *p53*. This haplotype harbours the D228E alteration in the protein sequence, which is therefore spread throughout a wide geographical range within this species' distribution. The shared haplotype with *M. lusitanicus* (haplotype 5) may have already been present in a common ancestral

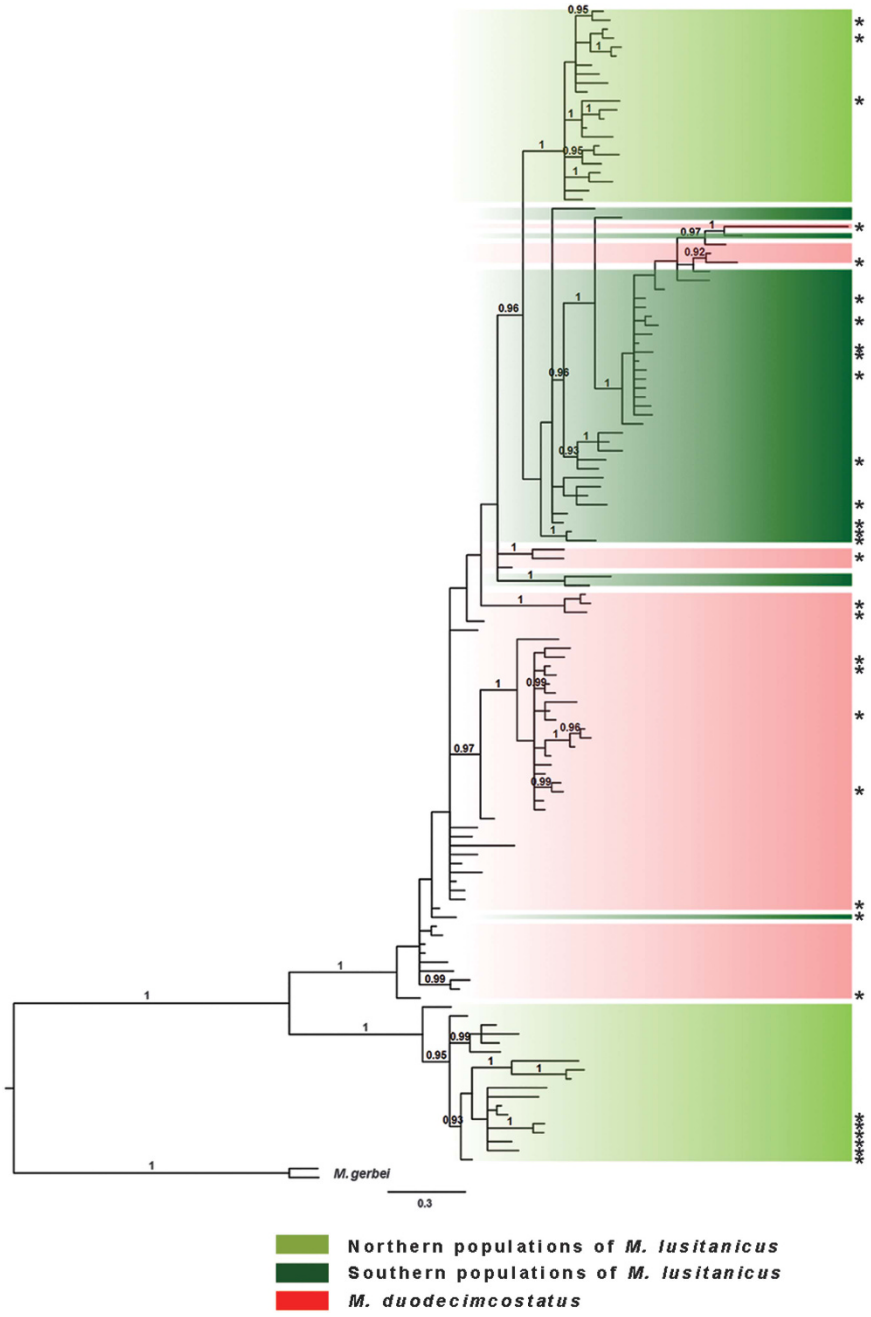


Figure 2 Phylogenetic relationships among *cytb* haplotypes of *M. lusitanicus* and *M. duodecimcostatus*. Phylogenetic tree was obtained with a Bayesian inference analysis and using *M. gerbei* as the outgroup. Numbers above branches represent posterior probabilities (only values >0.90 are shown). *Haplotypes corresponding to individuals on which sequencing of the *p53* gene was also performed.

species, persisting in *M. duodecimcostatus* by incomplete lineage sorting.

We found that *M. lusitanicus* may be separated in the two regional groups, one located northward and the other located southward of the Douro river, which possibly resulted from a range contraction of *M. lusitanicus* in the past. Haplotype richness and exclusiveness found in the northern populations of *M. lusitanicus* may be explained by the past relative isolation from the southern populations, whereas the lower haplotype and nucleotide diversities and ϕ_{ST} value found in the latter suggest that these populations suffered a more severe bottleneck followed by a rapid and recent expansion of an ancestral population with increase in effective population size and consequent spread of haplotype 5 south of the Douro river. The majority of the individuals from the northern region carried an extended tandem repeat sequence in intron 6, mostly in homozygosity. These additional repeats appear to have been independently formed on contemporary haplotypes—haplotypes 5, 6 and 21—with which present-day haplotypes unique to the northern region share single-nucleotide polymorphisms (compare Figure 1c and Supplementary Figure S2). Noteworthy, the tandem repeat region is located in close vicinity to the 5′ splice site of intron 6 of *p53* and is enriched in thymidines, making it a potential binding site for splicing regulators like TIA1 (Aznarez *et al.*, 2008). The majority of

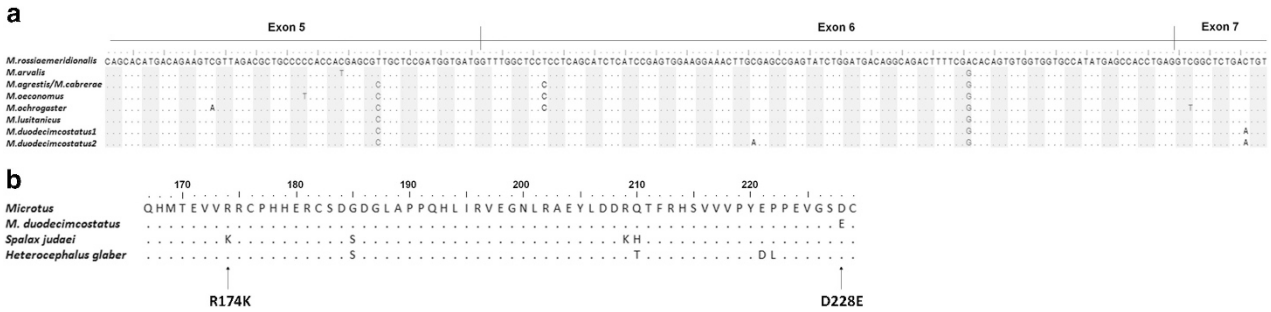


Figure 3 Comparison between *p53* exonic (exons 5 to 7) and protein sequences of *Microtus* and two subterranean species. (**a**) Alignment of *p53* exonic sequences found in *Microtus*. (**b**) Alignment of P53 protein sequences corresponding to *p53* exonic sequences of *Microtus*, *Spalax judaei* (accession number CAH03844) and *Heterocephalus glaber* (accession number EHB04497). Residue numbers are as in human P53 protein (two more residues than in *Microtus*). Mutations R174K occurring in *Spalax* and D228E from *M. duodecimcostatus* are indicated.

intron 6 polymorphic sites found in *M. lusitanicus* (5 out of 7) are present in the northern populations, suggesting that this gene region is mutation-prone in particular in these populations. The additional repeats could thus have evolved to prevent skipping of exon 6 in the presence of deleterious splice mutants, or appeared associated with a more mutagenic genomic background and remained in these populations as neutral mutations.

We propose that the secondary contact between *M. lusitanicus* and *M. duodecimcostatus* that resulted in the observed introgression of *M. duodecimcostatus* mtDNA into *M. lusitanicus* (Bastos-Silveira *et al.*, 2012; Barbosa *et al.*, 2013) presumably occurred before a range contraction of *M. lusitanicus*, as indicated by the presence of both non-introgressed and introgressed individuals in the northern populations and by the separation of introgressed individuals in the northern and southern populations (Figure 2). Introgression of the *p53* gene from *M. duodecimcostatus* into *M. lusitanicus* is not apparent from our results because of the absence in *M. lusitanicus* of the most frequent *p53* haplotypes found in *M. duodecimcostatus*, regardless of the *cytb* introgression status in *M. lusitanicus*. We cannot presently exclude the possibility of a generalized low level of nuclear introgression between these the two species due to the scarcity of the available data. Any significance for the non-introgression of *p53* needs to be further elucidated. Exclusion of *p53* from introgression could result from the incompatibility of the mutated *p53* within the *M. lusitanicus* genomic background.

In conclusion, our results provide suggestive evidence for the involvement of *p53* in the differentiation of the sister species *M. duodecimcostatus* and *M. lusitanicus* in the Iberian Peninsula, possibly via an adaptive role of the novel P53. Given the number of subcellular networks that involve P53 during stress responses, any alteration in this protein that becomes fixed, can potentially exert a pleiotropic effect by modifying more than one function of P53, and ultimately, through network rewiring and changes in gene expression patterns, organismal phenotype and fitness (Menendez *et al.*, 2007; Li and Johnson, 2010).

DATA ARCHIVING

Sequence data have been deposited in GenBank with accession numbers KR052208-KR052239. Additional information on each sampled specimen and *p53* haplotypes is available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.bc65p.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We are grateful to Isabel Rey from the Museo Nacional de Ciencias Naturales, Spain, for providing biological samples and to Margarida A. Duarte for assistance in data analysis. This work was supported by European Funds through COMPETE and by the National Funds through the Portuguese Science Foundation (UID/AMB/50017/2013 project, FCT-PTDC/BIA-BEC/103729/2008 project and FCT-SFRH/BPD/81925/2011 postdoctoral grant to ASQ).

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Supplementary Information accompanies this paper on Heredity website (http://www.nature.com/hdy)