



## Evidence of a complex phylogeographic structure in the common dormouse, *Muscardinus avellanarius* (Rodentia: Gliridae)

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This is the first mitochondrial phylogeography of the common dormouse, *Muscardinus avellanarius* (Linnaeus, 1758), a hibernating rodent strictly protected in Europe (Habitat Directive, annex IV; Bern Convention, annex III). The 84 individuals of *M. avellanarius*, sampled throughout the distributional range of the species, have been sequenced at the mitochondrial DNA gene (cytochrome *b*, 704 base pairs). The results revealed two highly divergent lineages, with an ancient separation around 7.7 Mya and a genetic divergence of 7.7%. Lineage 1 occurs in Western Europe (France, Belgium, and Switzerland) and Italy, and lineage 2 occurs in Central–Northern Europe (Poland, Germany, Latvia, and Lithuania), on the Balkan Peninsula, and in Turkey. Furthermore, these two lineages are subdivided into five sublineages genetically isolated with a strong geographical association. Therefore, lineage 1 branches into two further sublineages (Western European and Italian), whereas lineage 2 contained three sublineages (Central–Northern European, Turkish, and Balkan). We observed low genetic diversity within the sublineages, in contrast to the significant level of genetic differentiation between them. The understanding of genetic population structure is essential for identifying units to be conserved. Therefore, these results may have important implications for *M. avellanarius* conservation. © 2012 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2012, **00**, 000–000.

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## INTRODUCTION

Climatic oscillations during the Pleistocene have greatly affected the pattern of distribution of many species in the Western Palaearctic region, as well as their demographic history and patterns of population genetic differentiation (Avice, 2000; Hewitt, 2004). Three main peninsular refugia have been deduced from phylogenetic studies for most temperate species in Europe, namely Iberia, Italy, and the Balkans (Taberlet *et al.*, 1998; Hewitt, 1999, 2001; Michaux *et al.*, 2003; Krystufek, Bryja & Buzan, 2009). Increasing evidence suggests that the well-studied European southern and eastern refugia for thermophilous animal and plant taxa were supplemented by cryptic refugia in more northern Europe during the Late Pleistocene (Stewart & Lister, 2001). This hypothesis has received significant support with the discovery of northern refugia for small mammals (Jaarola & Searle, 2002, 2004; Brunhoff *et al.*, 2003; Deffontaine *et al.*, 2005; Kotlik *et al.*, 2006). In addition, some studies pointed to the role of Mediterranean refugia as sites of endemism (Bilton *et al.*, 1998; Stewart, 2003; Bhagwat & Willis, 2008; Provan & Bennett, 2008; Grill *et al.*, 2009; Krystufek *et al.*, 2009; Buzan *et al.*, 2010).

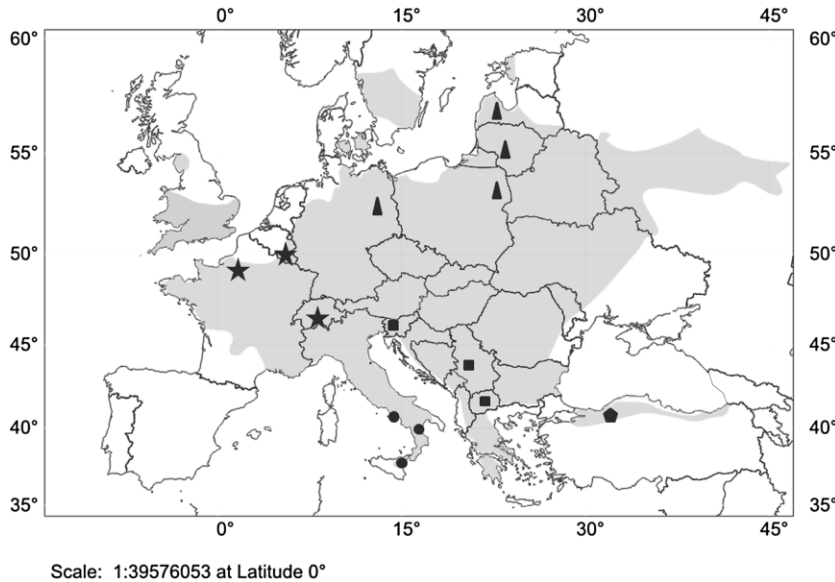
Nonetheless, all of these studies concern and give detailed phylogeographic patterns of species within the Muroid superfamily, whereas phylogeographic studies on Gliridae are almost non-existent, despite the interest in this group. Gliridae are one of the most ancient rodent families, emerging in the Eocene (between 54–37 Mya) (Nadachowski & Daoud, 1995). They are small- to medium-sized rodents, mostly arboreal, and were restricted throughout their history to Europe, Asia, and Africa (Wilson & Reeder, 2005). The diversification of the Gliridae began in the early Eocene, continued during the Oligocene, and culminated in the Late Early Miocene of Europe, where they appear to have occupied many ecological niches. The decline of this family becomes apparent during the Late Middle Miocene (Vallesian). Casanovas-Vilar *et al.* (2005) suggested that the diversity of forest-adapted rodents decreased significantly, not only in coincidence with the Vallesian climatic crisis, but also with the entry and spread of Muridae. Most of the 28 species contained in the family Gliridae (Holden, 2005) are now regarded as rare or endangered, attracting conservation-related research and active habitat management to assist their survival (Morris, 2003).

A recent phylogeographical study (cytochrome *b*) on the edible dormouse, *Glis glis* (Linnaeus, 1766), revealed three main haplogroups (Sicilian, Southern Italian, and European; Hürner *et al.*, 2010). This work evidenced a great genetic homogeneity in

Europe and a very low genetic diversity in the Mediterranean peninsulas. In this context, we feel that a clear understanding of the evolutionary history of the other members of the Gliridae family, obtained by comparative phylogeography, would allow the identification of biodiversity hot spots and increase awareness in conservation policies. In temperate regions, glirids are characterized by hibernation during winter, a behavior that has earned them a popular reputation for perpetual sleepiness (Nunome *et al.*, 2007). Thermal dependence during hibernation can constrain the biogeography of species, and therefore could imply another evolutionary history, especially during the Quaternary glaciations.

The target of our study is the common dormouse, *Muscardinus avellanarius* (Linnaeus, 1758), a Gliridae that is protected in Europe (Habitat Directive, annex IV; Bern Convention, annex III), and is included on the national Red Lists of many countries. *Muscardinus avellanarius* occurs in Europe and northern Asia Minor (Turkey) (Fig. 1). In continental Europe, it is fairly widespread, although it is absent from Iberia, south-west France, and boreal forests of the majority of Fennoscandia and Russia. It is also absent from the steppe landscapes of the eastern Ukraine and southern Russia. Island populations occur in southern Britain and on Corfu and Sicily (Morris, 1999; Rossolimo *et al.*, 2001). It is generally a lowland or mid-mountain species, although in the mountains *M. avellanarius* can reach an altitude of up to almost 2000 m a.s.l. (Kryštufek & Petkowski, 1990; Spitzenberger & Bauer, 2001; Juškaitis, 2008).

Population trends vary in different parts of the range: in some north-western areas (e.g. UK, the Netherlands, Denmark, and Belgium) populations are declining because of the interplay between the biological requirements of the species complex and habitat loss and fragmentation (Foppen, Verheggen & Boonman, 2002; Bright, Morris & Mitchell-Jones, 2006; Verbeylen, 2006; Mortelliti *et al.*, 2010). In others parts of the range, like Sweden and Lithuania, the species is considered to be stable (Juškaitis, 2008; Wretenberg & Berglund, 2009). It is probably an excellent model for studying the effects of habitat fragmentation, climatic shifts, and climatic stochasticity (Bright & Morris, 1996). In addition, *M. avellanarius* exhibits differences in hibernation in different ecogeographical conditions, confirming the peculiar link between climate and hibernation length (Sarà, Casamento & Spinnato, 2001; Panchetti *et al.*, 2004). Fossil data suggests that the Miocene/Pliocene boundary led to the diversification of the genus *Muscardinus* into several lineages, based on the different body sizes and dental morphologies (Storch, 1978; Aguilar, 1982; Nadachowski & Daoud, 1995; Aguilar & Lazzari, 2006; Alix *et al.*, 2008). Towards the end of



**Figure 1.** Geographical distribution of *Muscardinus avellanarius* in Europe and proximate location of the sampled populations. The shaded zone corresponds to the distribution area of the species. The symbols refer to lineages in Figures 2, 3, and 4, and in Tables 2, 3, and 4 (★, West European sublineage; ●, Italian sublineage; ■, Turkish specimen; ▲, Central–Northern European sublineage; ◆, Balkans sublineage).

the Pliocene most of these lineages vanished, and since the Middle Pleistocene only the extant species, *M. avellanarius*, survived in the European faunas (Nadachoswki & Daoud, 1995).

Incisive biological conservation of mammals or terrestrial vertebrates in Europe needs detailed data on the phylogeography, genetic diversity, and structure of the population of the species, as well as on the dynamics of past populations (Randi, 2003). The detection of phylogeographic structuring is important because it helps identify long-isolated populations that might have distinct gene pools and local adaptations; thus the conservation concern for *M. avellanarius* makes it an excellent candidate for such studies. We therefore subsequently defined the phylogeographic structure of the species, which has never yet been tackled. In particular we tried to answer the following questions: (1) is *M. avellanarius* structured phylogeographically; (2) how many distinct genetic lineages of *M. avellanarius* exist; (3) did the postglacial recolonization of Central Europe originate from the traditional Mediterranean refugia or from elsewhere; (4) did the Miocene/Pliocene boundary lead to the diversification of the genus *Muscardinus*; (5) what are the implications of our results for the conservation of the species?

## MATERIAL AND METHODS

### SAMPLE COLLECTION AND DNA EXTRACTION

We gathered a total of 83 sample tissues of *M. avellanarius* from 28 localities (between one and 12

samples per population) spread throughout the geographical range of the species (Fig. 1; Table 1). The specimens were obtained by the authors and their field collaborators (see the Acknowledgements). Tissues and hairs were preserved in 96% ethanol until DNA extraction. An additional sequence from Switzerland was downloaded from the GenBank database (Bentz & Montgelard, 1999). Total DNA was extracted using the DNeasy Tissue kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's instructions. DNA samples were extracted and amplified in a separate room solely dedicated to DNA extractions.

### PCR AMPLIFICATION AND MITOCHONDRIAL DNA SEQUENCING

A fragment of 704 bp was sequenced from cytochrome *b* (*cyt b*) of the mitochondrial DNA gene (mtDNA). PCR amplifications were carried out using primers designed by Andrea Grill specifically for *M. avellanarius*, modified from Bentz & Montgelard (1999): LMA14255, 5'-TGGTGGAAATTTTCGGTTCTCT-3'; RMA15192, 5'-GTTGGCCTCCAATTCATGTT-3'.

DNA isolated in some samples was highly degraded, and therefore the amplifications of the entire portion of the *cyt b* gene (> 700 bp) was unsuccessful. In order to recover this material, two further internally specific primers were designed by fragment alignment: MUSCAR\_RINTERN, 5'-AAGGTGAACT ATTACTAGGGC-3', combined with LMA14255 and

**Table 1.** Map references, geographic locations, corresponding sublineages, sample symbols, collectors, and GenBank accession numbers of *Muscardinus avellanarius* haplotypes used in this study

	Geographic origin	Sublineages	Total numbers of animals	Samples symbols	Haplotypes	Genbank accession number
Common dormouse ( <i>Muscardinus avellanarius</i> )	Macedonia Mount Galičica	Balkans	5	Ma1	Hap01, 04, 05	FN796753
						FN796756
Serbia	Popova Šapka Mount Pelister Mount Cer	Balkans	1	Ma2	Hap05	FN796757
						FN796758
						FN796759
						FN796760
Slovenia	Mount Krim	Balkans	2	Slo1	Hap03, 05	FN796761
						FN796762
Lithuania	Pogorelec Mount Kocevski Rog Šakiai district	Balkans	1	Slo2	Hap02	FN796763
						FN796764
						FN796765
						FN796766
Latvia		Central–Northern	12	Lit1	Hap06, 07, 08, 09	FN796767
						FN796768
Latvia		Central–Northern	3	Let1	Hap11, 12, 13	FN796769
						FN796770
Unknown Poland		Central–Northern	1	?	Hap 16	FN796771
						FN796772
Germany		Central–Northern	2	Po1	Hap10, 15	FN796773
						FN796774
Germany		Central–Northern	3	Ge1	Hap06, 14, 18	FN796775
						FN796776
Turkey	Mount Ulu dag	Turkish	1	Tur1	Hap17	FN796777
						FN796778
Switzerland	Canton de Vaud (Bentz & Montgelard, 1999)	West European	6	Swi1	Hap19	FN796779
						FN796780
France	Normandie	West European	1	Swi2	Hap22	AJ225117*
						FN796781
Belgium	Mechelen	West European	2	Fr1	Hap20	FN796782
						FN796783
Belgium		West European	6	Be1	Hap21	FN796784
						FN796785

Italy	Tevere Farfa(Lazio)	Italian	1	It10	Hap23	FN796774
	Castel di Guido (Lazio)	Italian	10	It7	Hap24, 27	FN796775
	Arcinazzo Romano (Lazio)	Italian	1	It8	Hap24	FN796776
	Viterbo (Lazio)	Italian	4	It11	Hap24, 26, 28	FN796777
	Filetino (Lazio)	Italian	1	It9	Hap25	FN796779
	Castelporziano (Lazio)	Italian	9	It6	Hap24, 27, 28	FN796776
	Perugia (Umbria)	Italian	2	It12	Hap24	
	Calabria	Italian	1	It3	Hap29	FN796780
	Cosenza (Calabria)	Italian	1	It4	Hap29	
	Catena Costiera (Calabria)	Italian	1	It5	Hap29	
	High Madonia (Sicily)	Italian	3	It2	Hap30, 32, 33	FN796781
						FN796783
						FN796784
						FN796782
						FN796783
Out-groups						
Garden dormouse ( <i>Eliomys quercinus</i> )						
	(Bentz & Montgelard, 1999)		1		AJ225030*	
	(Bentz & Montgelard, 1999)		1		FM16427*	
			1		FR848958	
			1		FR848957	
Asian garden dormouse ( <i>Eliomys melanurus</i> )						
			1		FR848955	
			1		FR848956	
Edible dormouse ( <i>Glis glis</i> )						
	(Hürner <i>et al.</i> , 2010)		1		FM16065*	

\*Accession numbers downloaded from the GenBank database.

MUSCAR\_LINTERN, 5'-ACCCTAGTAGAATGAATCTGA-3', combined with RMA15192. Those specific primers amplified two small overlapping *cyt b* fragments (300–400 bp), which were then aligned to give a sequence of 704 bp.

Amplifications were carried out following the protocol described by Michaux *et al.* (2003), and were performed in a Labover PTC100 thermal cycler employing 40 cycles (30–45 s at 94°C, 30–45 s at 50°C, and 45–90 s at 72°C), with a final extension at 72 °C (for 10 min). Products were visualized on an agarose gel to verify the success of amplification. All sequencing procedures were performed by Macrogen Inc. (Seoul, Korea). The sequences were edited and then aligned using the CLUSTALW algorithm in BIOEDIT 7.0.9.0 (Hall, 1999).

#### PHYLOGENETIC AND PHYLOGEOGRAPHIC ANALYSIS

The model of nucleotide substitution that best fits the data set was identified with the web application FINDMODEL, developed from MODELTEST (Posada & Crandall, 1998) and WEIGHBOR (Bruno, Socci & Halpern, 2000). A maximum-likelihood (ML) tree was constructed with PHYML 3.0, using the method described by Guindon & Gascuel (2003). The robustness of the trees was assessed by 1000 bootstrap replicates (Felsenstein, 1985).

The Bayesian phylogeny reconstruction (Yang & Rannala, 1997) was implemented in MRBAYES 3.1.1 (Huelsenbeck *et al.*, 2001). Bayesian posterior probabilities were picked from the 50% majority rule consensus of trees sampled every 1000 generations, discarding the trees obtained before the chains reached stationary distribution ('burn in', empirically determined by checking the likelihood values). A 50% majority-rule consensus tree was generated in PAUP 4.0b10 (Swofford, 2000).

A minimum spanning haplotype network was constructed using the MINSPNET algorithm available in ARLEQUIN 3.0 (Schneiders, Roessli & Excoffier, 2000), in order to more effectively portray the relationships among sequences for populations with low sequence diversity (Crandall & Templeton, 1993). To infer the relationships between haplotypes, a median-joining network was also constructed with the same combined sequence data set using NETWORK 4.5 (Bandelt, Forster & Röhl, 1999).

#### ANALYSIS OF GENETIC DIVERSITY AND DIFFERENTIATION

Haplotype (*h*) and nucleotide ( $\pi$ ) diversities (Nei, 1987), and their standard deviations (Tajima, 1989), and gene flow and genetic differentiation (using population pairwise  $F_{st}$  analysis) between the two major

lineages, and sublineages, were estimated using DNASP 5 (Librado & Rozas, 2009). The net distance between groups and average distances within groups were calculated in MEGA 4 (Tamura *et al.*, 2007).

The genetic structure of populations was examined using an analysis of molecular variance (AMOVA) performed in ARLEQUIN 3.0. AMOVA was conducted at three hierarchical levels of population subdivisions: among genetic groups (corresponding to the two lineages); among populations within each genetic group (corresponding to the sublineages); and within each population. The significance of these parameters was estimated by 10 000 random permutations of the distance matrix.

#### DIVERGENCE TIME

Relative rate tests and an approximate time of divergence between the observed mtDNA lineages were calculated as described in Michaux *et al.* (2003). The divergence time between *Eliomys quercinus* (Linnaeus, 1766) and *Eliomys melanurus* (Wagner, 1839) ( $7 \pm 0.9$  Myr; Montgelard, Matthee & Robinson, 2003) was used as a calibration point.

Another estimate of the divergence time of the main lineages of *M. avellanarius* used a Bayesian approach implemented in BEAST 1.5.4 (Drummond & Rambaut, 2007). We used the *E. quercinus/E. melanurus* divergence as a fossil point calibration. We included the entire set of mitochondrial sequences for the *Muscardinus* group as well as two sequences of *E. melanurus* (Table 1) and two sequences of *E. quercinus* (Table 1). Analyses were performed under the GTR + G substitution model parameter (previously estimated by FINDMODEL), an uncorrelated lognormal molecular clock, and a Bayesian Skyline plot demographic model (Drummond *et al.*, 2005). All other settings were defaults provided by BEAST. Two independent runs were performed, with 80 000 000 Markov chain Monte Carlo (MCMC) samples every 1000th generation. Results were visualized using TRACER 1.5.

#### ANALYSIS OF DEMOGRAPHIC HISTORY

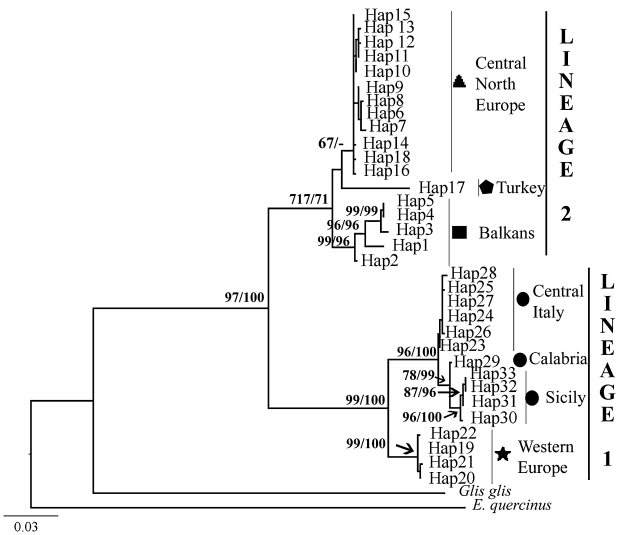
The hypothetical presence of glacial refugia was checked by searching for the possibility of population expansion. To avoid biased conclusions, we examined only the sublineages including more than 15 samples. We inferred past demographic trends for three sublineages: the Western, the Central–Northern, and the Italian sublineages. As the Italian sublineage is well structured in three groups, we also decided to infer the past trend for the central Italian group ( $N > 15$ ). Coalescent-based Tajima's *D* was calculated to test for selective neutrality (calculation using the total number of mutations; Tajima, 1989), and population

history was also inferred by testing departures from neutrality using  $R_2$  (Ramos-Onsins & Rozas, 2002) and Fu's  $F_S$  (Fu, 1996) in DNASP 5 (Librado & Rozas, 2009). Strobeck's  $S$  statistic (Strobeck, 1987) is the probability of having an equal number or fewer haplotypes than observed, based on the gene frequency distribution derived from the inferred mutation rate  $\theta$ . High  $S$  probability values (0.9–1.0) indicate a deviation from neutrality resulting from either selection or population expansion. Strobeck's  $S$  statistic was also calculated using DNASP 5 (Librado & Rozas, 2009). A Bayesian Skyline reconstruction performed in TRACER 1.5 allowed us to examine the historical demography of each lineage. We checked that the settings were clearly able to capture the model parameters. For example, an estimate of effective sample size (ESS) higher than 200 would indicate a good convergence of MCMC within chains, as suggested by Drummond & Rambaut (2007). The demographic histories of the sublineages of *M. avellanarius* were also examined using a mismatch distribution of pairwise nucleotide differences estimated in DNASP 5 (Librado & Rozas, 2009) for populations including more than 15 samples only. Multimodal mismatch distributions would correspond to a condition of demographic stability, whereas sudden population expansions would generate unimodal patterns (Slatkin & Hudson, 1991). The overall validity of the estimated demographic model was tested by obtaining the distribution of a test statistic, the sum of squared differences (SSD), between observed and expected mismatch distributions. A significant SSD value is considered as the evidence of departure from the estimated demographic model of a sudden population expansion using ARLEQUIN 3.0. Furthermore, we calculated the raggedness index (Harpending, 1994) of the observed mismatch distribution for each of the populations according to the population expansion model implemented in ARLEQUIN 3.0. Small raggedness values represent a population that experienced sudden expansion, whereas higher values of the raggedness index suggest stationary or bottleneck populations.

## RESULTS

### PHYLOGENETIC AND PHYLOGEOGRAPHIC ANALYSIS

A total of 33 haplotypes was identified among the 84 *M. avellanarius* specimens (Table 1). The total data matrix comprised 33 *Muscardinus* haplotypes. This matrix provided 704 bp, 120 of which were variable, 102 were parsimoniously informative, and 18 were singleton-variable sites. The average transitions/transversions ratio was 5935 and the nucleotide frequencies were: 28.2, 26.6, 13.7, and 31.4% for A, C, G, and T, respectively. The best model of sequence evo-



**Figure 2.** Maximum-likelihood tree for the 33 haplotypes of *Muscardinus avellanarius*. Numbers indicated on the branches correspond to bootstrap support obtained in the ML analysis (left) and Bayesian probabilities (right). Haplotypes origins are indicated in Table 1.

lution identified by the Akaike information criterion in FINDMODEL was the GTR + G model. The shape parameter of the Gamma distribution was 0.250. Three other sequences (two *E. quercinus* and one *G. glis*; Table 1) were chosen as out-groups according to a molecular phylogenetic study of Gliridae (Montgelard *et al.*, 2003).

The ML and Bayesian inference (BI) phylogenetic trees showed the same tree topology (Fig. 2). The haplotypes segregated into two lineages that gained strong support in ML (bootstrap support, BS: 97%) and in BI (Bayesian probability, BP: 100%). The *cyt-b* net genetic distance between these is very high (7.7%) (Table 2). The minimum spanning network (MSN) (Fig. 3) and the median joining network revealed a clear geographical partitioning of the haplotypes, with a considerable divergence between genomes occurring in different regions of the species range. They also reproduced exactly the same topology as ML and BI trees. The two major lineages are separated by 68 mutational steps.

The first lineage (hereafter lineage 1) split into two well-supported sublineages (BS 99%; BP 100%), the first of which encompassed individuals from Western Europe (Belgium, Switzerland, and France), whereas the second comprised all the haplotypes from Italy (including Sicily). These two sublineages are highly separated, with 28 mutational steps and a genetic net distance of 3.2%. The Italian sublineage further diverged into two supported groups (BS 96%; BP 100%), the first comprising specimens from

**Table 2.** Cytochrome *b* net genetic distance (NGD) in % between lineages

	Sample size ( <i>N</i> )	Balkans NGD	Central–Northern Europe NGD	Western Europe NGD	Central Italy NGD	Sicily NGD
All	84	–	–	–	–	–
Lineage 2	33	–	–	–	–	–
Balkans	11	–	–	–	–	–
Turkey	1	4	3.3	–	–	–
Central–Northern Europe	21	2.6	–	–	–	–
Lineage 1	51	–	–	–	–	–
Western Europe	15	8	7.7	–	–	–
Italy	36	–	–	–	–	–
Central Italy	28	8.6	8.6	3.2	–	–
Sicily	5	8.1	8.1	4	1.2	–
Calabria	3	8.4	8.5	3.4	0.8	0.7

central Italy and the second comprising specimens from southern Italy. The latter was further split into two subgroups (a Sicilian and a Calabrian group), which found support only in BP (100%). These groups were separated by six mutational steps between each of them, and a net distance of 0.8 and 0.7%, respectively, between central Italian and Calabrian groups and between Calabrian and Sicilian groups.

The second major lineage (hereafter lineage 2) gathers populations from the remainder of the species range, in Central–Northern Europe (Lithuania, Latvia, Germany, and Poland), the Balkan Peninsula (Macedonia, Slovenia, and Serbia), and Turkey. The Balkans sublineage and the single Turkish haplotypes are separated by 17 and 28 mutational steps, respectively, from the Central–Northern European sublineage, and by a net distance of 2.6 and 3.3%. A further substructuring was poorly evident in lineage 2; however, the Balkan samples may hold a sister position against those from Central–Northern Europe.

#### ANALYSIS OF GENETIC DIVERSITY AND DIFFERENTIATION

The AMOVA analysis showed that 70.43% ( $P = 0.000$ ) of the total mtDNA variation was distributed between the two genetic groups: 26.30% ( $P = 0.000$ ) among populations within groups, and 3.26% ( $P = 0.000$ ) within populations.

Our results are summarized in Table 3 and generally indicate a very low level of diversity for the two lineages (lineages 1 and 2), with a  $\pi$  value of 0.02. The highest nucleotide diversities were found in the Balkan and the Italian sublineages, with  $\pi$  values of 0.00668 and 0.006 respectively.  $F_{ST}$  values (Table S1,

Supporting Information) are very high and significant among all the sublineages.

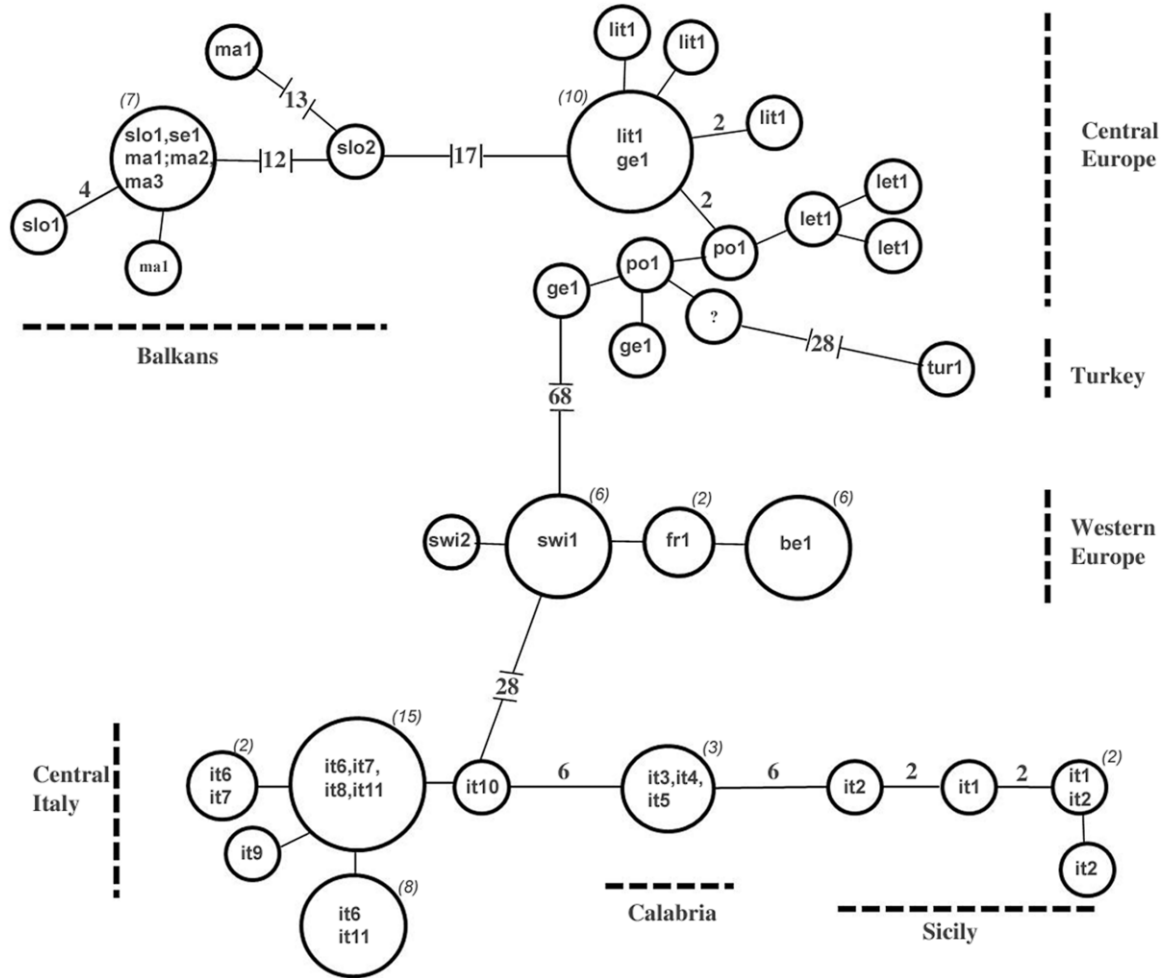
#### DIVERGENCE TIME

The relative rate did not show any difference of evolutionary rate among the observed lineages. This allowed us to apply a molecular clock so that the approximate time of divergence between the observed mtDNA sublineages could be calculated.

According to the mean Kimura two-parameter (K2P) distance between *E. quercinus* and *E. melanurus*, the gross estimate of the evolutionary rate for the Gliridae is around 1% per Myr. The application of this rate to the different dichotomies obtained within the *M. avellanarius* tree resulted in the following approximate molecular dating: 7.7 Myr for the split between lineages 1 and 2 (10.81 Myr with BI); 3.2 Myr between the Western sublineage and the Italian sublineage (3.53 Myr with BI) and between the Central–Northern sublineage and the Turkish sample (2.71 Myr with BI); and 2.6 Myr between the Balkan sublineage and the Central–Northern sublineage (3.9 Myr with BI). Finally, the separation between the Central Italian and the Calabrian groups, and between the Calabrian and the Sicilian groups, should have taken place around 0.8 and 0.7 Mya, respectively (1.19 Myr between the Central Italian and the Calabrian–Sicilian groups with BI).

Therefore, divergence times calculated using the coalescent approach were similar to the K2P-corrected distances, except for the divergence time between the two lineages. These values are summarized in Figure 4. Because of the wide uniform priors we used

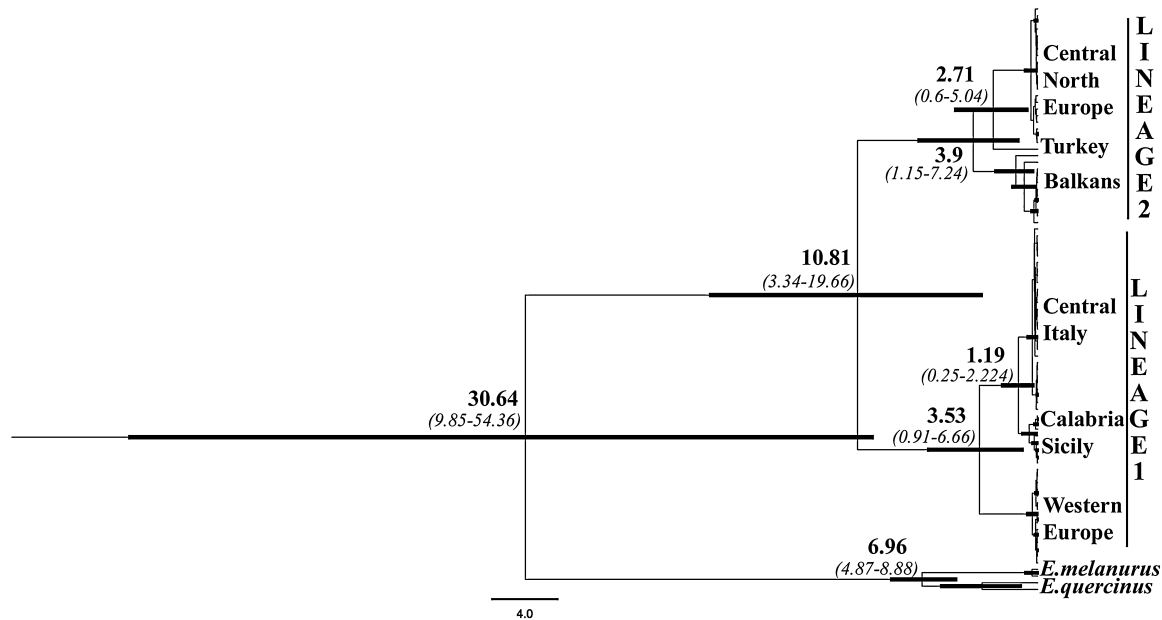




**Figure 3.** A minimum spanning network constructed using the 33 haplotypes of mitochondrial cytochrome *b* gene sequences. Geographic origins (Table 1) are noted. Numbers correspond to the mutational steps observed between haplotypes, numbers in parentheses correspond to the number of animals presenting this haplotype, and the size of the circle is proportional to the numbers of haplotypes represented.

**Table 3.** Summary of haplotypes (Hd) and nucleotide diversity (Pi), and their standard deviations, observed within the main genetic groups of *Muscardinus avellanarius*

	Sample size ( <i>N</i> )	Pi (± SD)	Hd (± SD)
All	84	0.05992 ± 0.00193	0.921 ± 0.015
Lineage 2	33	0.0211 ± 0.00262	0.841 ± 0.049
Balkans	11	0.00668 ± 0.00294	0.491 ± 0.175
Turkey	1	–	–
Central–Northern Europe	21	0.00337 ± 0.00707	0.786 ± 0.096
Lineage 1	51	0.0225 ± 0.00	0.844 ± 0.00124
Western Europe	15	0.00168 ± 0.00013	0.705 ± 0.074
Italy	36	0.006 ± 0.00125	0.742 ± 0.064
Central Italy	28	0.00151 ± 0.00024	0.566 ± 0.079
Sicily	5	0.002 ± 0.00081	0.7 ± 0.218
Calabria	3	0.00095 ± 0.00045	0.667 ± 0.314



**Figure 4.** Maximum probability tree derived from BEAST analysis. Numbers in bold indicate the posterior mean estimates of divergence time. Node bars and numbers in italic illustrate the width and the value of the 95% highest posterior density (HPD).

**Table 4.** Results of neutrality tests (Tajima's  $D$ , Fu's  $F_s$ ,  $R_2$ ) and mismatch analyses for the three sublineages and the Central Italian group ( $N > 15$ )

	Sample size ( $N$ )	Tajima's $D$	Fu's $F_s$	$R_2$	Strobeck's $S$	Raggedness index, $R_g$	Mismatch distribution, SSD
All	84	–	–	–	–	–	–
Lineage 2	33	–	–	–	–	–	–
Balkans	11	–	–	–	–	–	–
Turkey	1	–	–	–	–	–	–
Central–Northern Europe	21	–0.507	–6.318*	0.107	1*	0.09014	0.03325
Lineage 1	51	–	–	–	–	–	–
Western Europe	15	0.833	0.034	0.193	0.751	0.10204	0.02245
Italy	36	0.218	0.634	0.285	0.509	0.09384	0.04341
Central Italy	28	–0.484	–0.552	0.105	0.827	0.240	0.05832
Sicily	5	–	–	–	–	–	–
Calabria	3	–	–	–	–	–	–

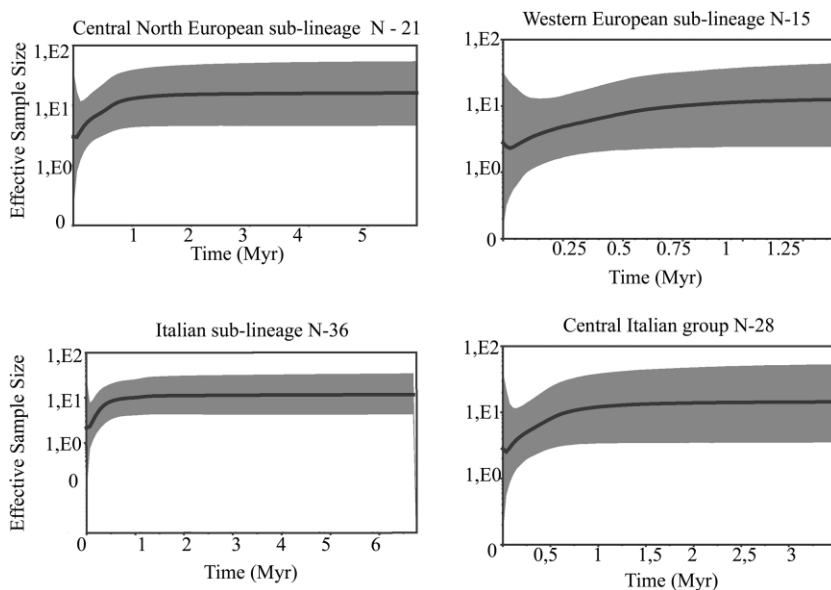
\* $P < 0.05$ .

for the calibration points, the confidence interval (3.34–19.66 Mya) remains large, so we decided to use the value of the substitution rate between the two lineages.

#### ANALYSIS OF DEMOGRAPHIC HISTORY

Neutrality tests of Tajima's  $D$  revealed non-significant values in all populations (Table 4). Furthermore, the

$R_2$  values fall within the range expected under the model of constant population size, thereby accepting the null hypothesis of constant population size in all populations. Non-significant Strobeck's  $S$  and negative Fu's  $F_s$  values were obtained for the Western, Italian, and central Italian sublineages. However, Fu's test for neutrality (Fu, 1996) and the Strobeck's  $S$ -test indicated a deviation from neutrality, resulting either from selection or from population expansion for the data set



**Figure 5.** Bayesian skyline plots of the historical demography of *Muscardinus avellanarius*. Time is shown in million years to the present day (= 0). Demographic trends for the Central–Northern European, Italian, and Western European sublineages, as well as for the central Italian group, are shown. Solid lines and shaded areas represent the median estimates and the 95% highest posterior density (HPD).

of the Central–Northern European lineage ( $F_s = -6318$ ,  $P = 0.000$ ;  $S = 1$ ,  $P = 0.001$ ).

The Bayesian skyline plot reconstruction (Fig. 5) showed that the four lineages appear to have experienced a long period of constant population size, followed by a decline that started around 1 Mya. Also, the mismatch distribution for the four clusters above did not show the bell-shaped curve that is consistent with the hypothesis of rapid population expansion (Fig. 6). The mismatch population test statistics SSD and raggedness index values were also consistent with constant population sizes (Table 4).

## DISCUSSION

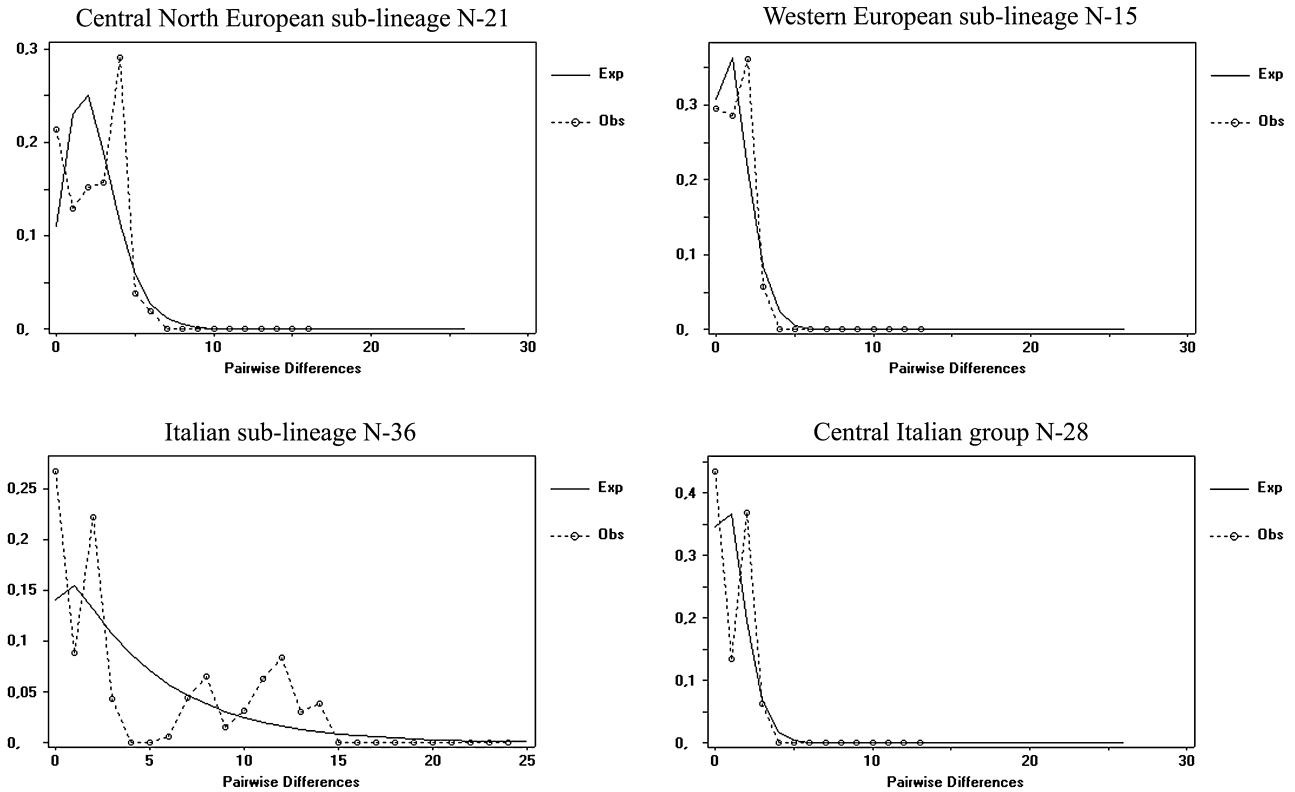
### GENETIC STRUCTURE AND TIME OF DIVERGENCE OF *MUSCARDINUS AVELLANARIUS*

Consistent with palaeontological data and the resulting 7-Myr period of divergence, we hypothesized that the putative ancestors of *M. avellanarius* (*Muscardinus hispanicus* de Bruijn, 1966 and *Muscardinus pliocaenicus* Kowalski, 1963; Garcia-Alix *et al.*, 2008) must have split from one another very early, and subsequently by allopatry became two highly divergent genetic lineages in Europe.

Our mtDNA study suggests that the Late Miocene/Early Pliocene was an important period for the diversification of European mammals. Several other Western and Eastern European mammalian taxa are known to have diverged at about the same time, such

as *Talpa* spp. (Colangelo *et al.*, 2010), *Erinaceus europaeus* Linnaeus, 1758 and *Erinaceus roumanicus* Barrett-Hamilton, 1900 (Santucci, Emerson & Hewitt, 1998), or the eastern and western clade of *Cervus elaphus* Linnaeus, 1758 (Ludt *et al.*, 2004). A closer sampling in the possible zones of overlap between the two ancient lineages could reveal a contact zone, as shown in previous studies, e.g. for yellow-bellied and fire-bellied toads [*Bombina variegata* (Linnaeus 1758) and *Bombina bombina* Linnaeus 1761; Szymura, 1993], oaks (*Quercus robur* group; Ferris, Oliver & Davy, 1993), and shrews (*Sorex araneus* Linnaeus, 1758 group Taberlet, Fumagalli & Hausser, 1994).

During the Late Pliocene, and throughout the Quaternary, a substantial subdivision of extant *M. avellanarius* lineages into further sublineages seems to have occurred. Based on the results and on our estimation of divergence time, lineage 1 split into Western European and Italian sublineages around 3.2 Mya. Lineage 2 split into Central–Northern European, Turkish, and Balkan sublineages around 3.2 Mya. Coincident with these divergence times around 3 Mya at the Pliocene–Pleistocene boundary, a further strong climatic deterioration occurred with the intensification of glaciations and the establishment of the great northern ice sheets in America and in Europe (Santucci *et al.*, 1998). The Early Pleistocene saw the definitive decline of Tertiary forests in north-western Europe (West, 2000), and



**Figure 6.** Mismatch distribution of cytochrome *b* sequences (704 pb) for the Central–Northern European, Western European, Italian sublineages, and the central Italian group.

the disappearance of such rich floristic habitats might have promoted the further isolation of *M. avellanarius* lineages.

#### PAST DEMOGRAPHY

The generalized Bayesian Skyline reconstruction showed that the sublineages have experienced a long period of constant population size, followed by a general decline that started around 1 Mya. The fragmentation of the habitat, leading to a contraction of the effective size of the habitat area, the intensification of glaciations, and competition with the Muridae family could explain the demographic decline observed since the Early Pleistocene (1 Mya). This is further evidenced by the neutrality tests (mismatch analysis), as none of the mismatch graphics showed a bell-shaped curve that would indicate a population expansion. The only exception concerns the Central–Northern European sublineage, where Strobeck's *S*-test, the star-like topology of the network, and Fu's  $F_S$  values all indicate a signal of population expansion. There is no clear evidence of recent expansion in the Bayesian plot for the Central–Northern European sublineage, as the confidence interval can reveal either an expansion or a decline.

#### REFUGIA AND POSTGLACIAL RECOLONIZATIONS

The phylogeographic analysis allowed the reconstruction of refugia for *M. avellanarius*, although in some cases more sampling and additional analysis is needed (e.g. Central–Northern European sublineage, Balkan sublineage, Turkish specimen).

#### *Italian sublineage*

The high level of genetic diversity (Table 3) indicates that the Italian region was one of the refugia for *M. avellanarius*. Furthermore, the mismatch analysis and the neutrality tests suggest the existence of a stable population in the Italian region. During Quaternary glaciations this sublineage was able to survive the general cooling, and diversified genetically in different parts of the peninsula. This led to the appearance of three genetic groups corresponding to the following regions: Central Italy, Sicily, and Calabria. This separation is confirmed by the  $F_{ST}$  values, which are very high and are significant among the three groups (Supporting information). These groups are probably the result of geographic isolations in three different refugia, associated with the fragmentations of forests that appeared during Quaternary cold stages (Magri *et al.*, 2006). The long-term

isolation of Sicily and southern Calabria from the rest of Italy resulted from the marine-flooded graben in central Calabria, which appeared for most of the Pleistocene, as attested by the presence of several endemic plants (Pignatti, 1982) and animals in these regions (Malatesta, 1985; Caloi, Malatesta & Palombo, 1989; Santucci, Nascetti & Bullini, 1996; Canestrelli *et al.*, 2010). Our approximate time of divergence, with a separation between the three groups around 1 Mya, seems to corroborate such biogeographic scenarios. This result strongly suggests the possible existence of refugia within refugia in the Italian peninsula, as has already been observed for several other species (Michaux *et al.*, 2003; Canestrelli *et al.*, 2006, 2007, 2008; Castiglia *et al.*, 2007; Grill *et al.*, 2009; Vega *et al.*, 2010).

#### *Western sublineage*

The high endemism of the Italian sublineage strongly suggests that such a population did not contribute to the postglacial recolonization of Western Europe. This implies that *M. avellanarius* living in France, Belgium, and Switzerland, and now grouping in the Western sublineage, had other refugia outside of the 'traditionally accepted' refugia. There were areas situated outside the permafrost during the maximum cooling period (Sommer & Nadachowski, 2006) that were within the current range of the Western sublineage, e.g. the area of the Dordogne in south-western France.

#### *Balkan sublineage*

The high level of genetic diversity (Table 3) indicates the Balkan region was another glacial refuge for *M. avellanarius*. The Balkan Peninsula is topographically the most diverse landscape in Europe (Reed, Krystufek & Eastwood, 2004), and such variability could have provided a suitable environment for altitudinal shifts in response to climatic change during glacial–interglacial oscillations, and also for small-scale allopatric isolation (Krystufek *et al.*, 2007). This latter hypothesis could be confirmed by the high number of mutation steps within the Balkan sublineages in the minimum spanning network (Fig. 3). Recent studies suggest multiple glacial refugia in the Balkan Peninsula for different species, such as *Dinaromys bogdanovi* (Martino, 1922) (Krystufek *et al.*, 2007), *Rana (Pelophylax)* (Lymberakis *et al.*, 2007), and *Spermophilus citellus* (Linnaeus, 1766) (Krystufek *et al.*, 2009).

#### *Central–Northern European sublineage*

The star-like topology in the minimum spanning network, the Fu's  $F_s$  value, and the Strobeck's  $S$  index suggest a rapid expansion/colonization event for the Central–Northern European sublineage. However,

the refugium of this sublineage is unclear. All analyses (Maximum Likelihood, Minimum Spanning Network, genetic distance analysis, and  $F_{ST}$  value) showed that the Balkan sublineage is highly divergent from the Central–Northern European sublineage. These results tend to imply that the Balkan sublineage did not contribute to the colonization of Central–Northern Europe. As suggested for several other species (Seddon *et al.*, 2002; Deffontaine *et al.*, 2005; Kotlik *et al.*, 2006), modern populations of *M. avellanarius* from Central–Northern Europe could be derived from populations that survived in the Carpathian region, as it was covered with patches of mixed coniferous and deciduous forests instead of a uniform steppe-like landscape (Willis, Rudner & Sümegi, 2000). Fossil data tend to suggest such a hypothesis. Indeed, numerous fossil records of *M. avellanarius* have been found during the Late Vistulian (Pleistocene) period in the Deszczowa Cave in southern Poland (Nadachowski, 1989).

#### *Turkish sublineage*

The single Turkish specimen is also highly divergent compared with the Central–Northern European specimens. During the last glacial maximum, temperate forest remained in northern Turkey in a narrow band along the southern shore of the Black Sea, with a patchy extension to the south-west Caucasus (Adams & Faure, 1997). Pollen records indicate that deciduous oak was present in the southern, and particularly south-western, part of the Caucasus region from at least 12 000 years BP (Huntley, 1990, 1992). It seems possible therefore that this area of northern Turkey could be the site of a glacial refuge (Seddon *et al.*, 2002). This hypothesis would need to be confirmed by a more extensive sampling of this region.

#### TAXONOMIC AND CONSERVATION IMPLICATIONS

The genetic divergence between lineage 1 and lineage 2 (approximately 7.7%) falls within the range of inter- and intraspecific *cyt b* distances observed in mammals (Bradley & Baker, 2001), more specifically in the Arvicolinae (Conroy & Cook, 2000; Jaarola & Searle, 2002, 2004; Haynes, Jaarola & Searle, 2003) and in the Glirinae (Bentz & Montgelard, 1999). Thus, the West European and Italian populations could be described under the phylogenetic species concept (Cracraft, 1983), like a subspecies or even an allo-species separated from a second subspecies formed by the Central–Northern European, Balkan, and Turkish populations. However, such a phylogenetic approach based on only one genetic marker is no longer widely accepted (Avice & Ball, 1990). Additional data from genetically independent loci is required before solid taxonomic conclusions can be made.

Corbet (1978) tentatively recognized five subspecies of *M. avellanarius* based on the morphological studies of Witte (1962) and Roesler & Witte (1969: *M. a. avellanarius* (including *anglicus*, *corilinum*, and *muscardinus*) in Sweden; *M. a. kroeckii* Niethammer & Bohmann, 1950 in Bulgaria; *M. a. pulcher* Barrett-Hamilton, 1898 (including *niveus* and *speciosus*) in Italy and Sicily; *M. a. zeus* Chaworth-Musters, 1932 in Greece; and *M. a. trapezius* Miller, 1910 in Asia Minor. Namely, the geographic variation has never been assessed throughout the range of the species, and discontinuities in morphological variation have not been demonstrated. A more recent view observed great morphological homogeneity among the European populations, which does not allow the delineation of different subspecies (Wilson & Reeder, 2005). According to our results, there is no congruence between our genetic lineages and the five previously recognized subspecies. However, the present genetic study would affirm that *M. avellanarius* could be composed of a minimum of five historically isolated mitochondrial lineages. Even if taxonomic conclusions cannot be made, those lineages could be regarded as independent units for conservation management purposes (Hillis, Moritz & Mable, 1996; Kocher & Stepien, 1997). The high genetic divergence between all the lineages and the ancient separation of the lineages reflects low mobility. The conservation of such evolutionary significant units (ESUs) is regarded as an important goal for preserving species (Moritz, 1994, 1999).

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

**Table S1.** Pairwise  $F_{ST}$  between lineages. \* $P < 0.5$ .

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