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Mitochondrial phylogeography of the Woodmouse (*Apodemus sylvaticus*) in the Western Palearctic region

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Abstract

We sequenced 965 base pairs of the mitochondrial DNA cytochrome *b* from 102 woodmice (*Apodemus sylvaticus*) collected from 40 European localities. The aims of the study were to answer the following questions. (i) Did the Mediterranean peninsulas play a role as refuge for woodmice? (ii) Is genetic variability of *A. sylvaticus* higher in the Mediterranean region compared with northern Europe? (iii) Are the patterns of the postglacial colonization of Europe by woodmice similar to those presently recognized for other European species? The results provide a clear picture of the impact of the Quaternary glaciations on the genetic and geographical structure of the woodmouse. Our analyses indicate a higher genetic variability of woodmice in the Mediterranean peninsulas compared to northern Europe, suggesting a role of the former as refuge regions for this small mammal. An original pattern of postglacial colonization is proposed where the Iberian and southern France refuge populations colonized almost all European regions. The Sicilian population appears to be very differentiated and highly variable. This emphasizes the importance of this island as a 'hot spot' for the intraspecific genetic diversity of the woodmouse. Finally, woodmice in North Africa originated from southwestern Europe, most probably as a result of a recent anthropogenic introduction.

Keywords: *Apodemus sylvaticus*, glacial refuge, mtDNA, North Africa, phylogeography, postglacial colonization, Western Palearctic region

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Introduction

An increasing number of studies on phylogeographic patterns in European species of plants (i.e. Sharble *et al*. 2000; Widmer & Lexer 2001) and different groups of animals such as insects (i.e. Lunt *et al*. 1998; Mardulyn 2001), amphibians (Szymura *et al*. 2000), fish (Alexandrino *et al*. 2000; Engelbrecht *et al*. 2000; MacHordon *et al*. 2000), birds (Lucchini & Randi 1998; Pitra *et al*. 2000) and several mammals (Bilton *et al*. 1998; Santucci *et al*. 1998; Seddon *et al*. 2001) were recently published.

They have shown the value of molecular genetic data in understanding the effects of Quaternary climatic changes on the intraspecific diversity of European species. During the last 2.4 millions years (Myr), temperate species underwent several contractions and expansions of range. This resulted in extinctions of the northern populations during ice ages followed by subsequent northward expansions from southern refugia (mainly the Mediterranean peninsulas) during interglacials (Taberlet *et al*. 1998). Some major effects on the genetic structure of such environmentally induced range changes were appreciated by Avise (1994) and Hewitt (1996). They hypothesized that rapid expansion from refugial populations involved serial bottlenecking with progressive loss of allelic diversity, resulting in less genetic diversity among populations present in the more recently colonized places. In contrast, it is expected that populations living in the Mediterranean refuge regions were less affected by climatic changes and will be more genetically diversified. This has been observed in the European greenfinch (*Carduelis chloris*) (Merilae *et al*. 1997).

Moreover, the isolation of populations of many species in separate southern regions during ice ages led to the

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allopatric differentiation of several genetic groups, which recolonized the Western Palearctic region at the end of the last ice age, 16 000 years ago. On the basis of the present distribution of different genetic groups all over Europe, Hewitt (1999, 2001) described three patterns of postglacial colonization. In the first one (*Chorthippus parallelus* pattern), the postglacial expansion originated only from the Balkans. In the second (*Erinaceus europaeus* pattern), the species colonized northern Europe from three refuge regions: the Iberian peninsula, Italy and the Balkans. Finally, in the third one (*Ursus arctos* pattern), the species appeared to have colonized most of Europe from two refugia: the Iberian peninsula and the Caucasus/Carpathians.

These studies provide valuable information on the phylogeography of European species. However, several aspects of the influence of the Quaternary climatic fluctuations on the geographical distribution of genetic diversity in Europe remain unclear, particularly for some groups of animals such as small mammals. Firstly, with the exception of the study on hedgehogs (*Erinaceus europaeus* and *E. concolor*) (Seddon *et al*. 2001) and some data on the grasshopper *C. parallelus* (Hewitt 1999), scant information is available on the level of genetic diversity of animals, particularly comparing southern European regions to northern ones. This kind of information could be very valuable from a conservation biology perspective. Secondly, are recolonization patterns other than those described by Hewitt (1999) likely? Finally, with the exception of the study by Seddon *et al*. (2001), the studies performed on European small mammals either: (i) did cover the entire distribution range of the species but were based on restricted samples (Taberlet *et al*. 1998 for *Crocidura* and *Arvicola*; Bilton *et al*. 1998) or (ii) analysed a restricted part of the distribution areas of the species (Taberlet *et al*. 1994; Jaarola & Tegelström 1995). This lack of data prevents the drawing of any definitive conclusion concerning the role of the Mediterranean peninsulas as refuges for these species. Indeed, Bilton *et al*. (1998) proposed that widespread small mammals did not recolonize northern Europe from the Mediterranean peninsulas, which in turn represent 'factories of allopatric speciations'.

The aim of our study is to address these questions in studying a small rodent species, the woodmouse (*Apodemus sylvaticus*). This species has several advantages for a phylogeographical study. The woodmouse is relatively common, easy to catch and widespread all over the Western Palearctic region. It is a forest species present in the Western Palearctic region for the past 3 Myr. (Michaux & Pasquier 1974). Therefore, like the forests, it probably survived during the Quaternary glaciations through important fluctuations in its distribution area.

Moreover, the evolutionary history of the genus *Apodemus* is well known through palaeontological (Michaux & Pasquier 1974; Michaux *et al*. 1997) and molecular phylogenetic (Serizawa *et al*. 2000; Michaux *et al*. 2002) studies.

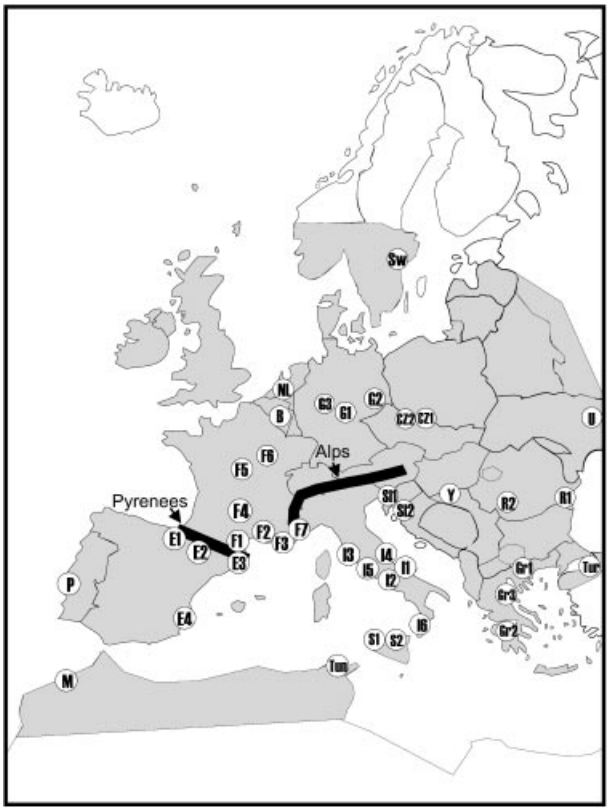

Fig. 1 Geographical distribution of the *Apodemus sylvaticus* samples. The shaded zone corresponds to the distribution area of the species (as described by Schilling *et al*. 1986; Zagorodnyuk 1996; and Mezhzherin 1997). Thick lines correspond to the main European biogeographic barriers: the Pyrenees and the Alps. See Table 1 for sample symbols.

Therefore, a calibration of its molecular clock is possible. This will allow the verification of the importance of the Quaternary ice ages in a possible intraspecific differentiation within *A. sylvaticus.*

Finally, the woodmouse is also distributed in other regions, such as northern Africa or the Mediterranean islands (Fig. 1). The genetic study of specimens from these regions will give new information on their geographical origin.

Materials and Methods

A total of 102 *Apodemus sylvaticus* taken from 40 localities throughout its geographical range have been analysed (Table 1, Fig. 1).

DNA was extracted from ethanol-preserved tissue as described by Sambrook *et al*. (1989). These tissues were taken from the *Apodemus* tissue collection of J. R. Michaux

Table 1 Geographic distribution and references of *Apodemus* tissues used for the experiments

Geographic origin		Total no. of animals	Sample symbols (see Figs 1, 2 and 3)	Tissue sample or GenBank accession numbers (for sequenced samples)
Apodemus sylvaticus				
Spain	Eugi (Navarre)	4	E1	T-2141, T-2142, T-2143, T-2144
	Torla	1	E2	JRM-700
	Figueras (Catalogna)	1	E3	JRM-578
	Murcia	4	E4	JRM-594, JRM-596, JRM-714, JRM-715
Portugal	Murtal	2	P	JRM-563, JRM-564
France	Py Mantet (Pyrénées orientales)	7	F1	JRM-269, JRM-270, JRM-271, JRM-272, JRM-274 JRM-277, JR -278
	Murviel (Hérault)	3	F2	JRM-574, JRM-575, JRM-577
	Cap Lardier (Var)	4	F3	JRM-142 to JRM-145
	Sérandon (Corrèze)	1	F4	JRM-396
	Allier	1	F5	T-665
	Morvan	1	F6	JRM-589
	Alpes Maritimes	1	F7	JRM-584
Italy	Penne (Abruzzo)	4	I1	JRM-163, JRM-164, JRM-165, JRM-167
	Rome (Latium)	1	I2	JRM-168
	Grosseto (Toscania)	1	I3	JRM-176
	San Polo (Latium)	1	I4	JRM-171
	Tarquinia (Toscania)	3	I5	JRM-172, JRM-173, JRM-174
	Aspromonte (Calabria)	4	I6	JRM-159 to JRM-162, JRM-851 to JRM-860
Sicily	Alia (Center Sicily)	3	S1	JRM-304 to JRM-306
	Madonia Forest (northern Sicily)	12	S2	JRM-868 to JRM-879
Belgium	Ardennes	7	B	JRM-101, JRM-102, JRM-103, JRM-104, JRM-105, JRM-106, JR M-107
Netherlands		1	NL	ABO33695
Germany	Dresden (Saxony)	2	G1	JRM-515, JRM-519
	Leipzig (Saxony)	1	G2	JRM-514
	Bielefeld	1	G3	JRM-157
Czech Republic	Kasperske	3	CZ1	JRM-372 to JRM-374
	Klinec jiloviste	1	CZ2	JRM-375
Sweden	Uppsala	3	Sw	JRM-452, JRM-453, JRM-455
Yugoslavia	Susarra (Vojvodine)	3	Y	JRM-210 to JRM-212
Slovenia	Skofja	1	Sl1	JRM-417
	Ljubljana (Vecnapot)	2	Sl2	JRM-434, JRM-436
Ukraine	Tchernobyl	4	U	AF127536, 37, 38, 43
Greece	Macedonia	1	Gr1	JRM-181
	Péloponnèse	1	Gr2	JRM-183
	Central Greece	1	Gr3	JRM-705
Romania	Cheile Garlistei	1	R1	JRM-570
	constanta county	1	R2	JRM-571
Turkey	Caycuma (Zonjuldak)	1	Tur	JRM-184
Tunisia	Ain Dram	4	Tun	JRM-138 to JRM-141
Morocco	Ket ama	4	M	JRM-138 to JRM-141
Apodemus flavicollis				
Italy	Abruzzos	1		AJ311150
France	Allier	1		AJ311151
Apodemus mystacinus				
Syria		1		AJ311146
Greece		1		AJ311147
Apodemus agrarius				
Estonia		1		AJ311144
Czech Republic		1		AJ311145

(JRM-numbers) and the mammal tissue collection housed at the Institut des Sciences de l'Evolution de Montpellier (Catzeflis 1991; T-numbers).

By modifying the universal polymerase chain reaction (PCR) primers L7 (5'-ACCAATGACATGAAAAATCATCGTT-3') and H16 (5'-ACATGAATYGGAGGYCAACCWG-3'), originally described by Kocher *et al*. (1989), 965 base pairs of cytochrome *b* were amplified. Amplification reactions were carried out in 2 × 50 μL volumes including 25 μL of each 2 μM primer, 20 μL of 1 mM dNTP, 10 μL of 10× reaction buffer, 10 μL of purified water and 0.2 μL of 5 U/μL Promega *Taq* DNA polymerase. Approximately 200 ng of DNA extract (10 μL) was used per PCR amplification. Amplifications were performed in a Labover PTC100 thermal cycler employing 33 cycles (20 s at 94 °C, 30 s at 50 °C and 1 min 30 s at 68 °C) with a final extension cycle of 10 min at 68 °C. PCR products were purified using the Ultra-free DA Amicon kit (Millipore) and directly sequenced. Both strands were sequenced using a Bigdye terminator (Applied Biosystems) sequencing kit on an ABI 310 (Applied Biosystems) automated sequencer.

Sequence alignment and saturation analysis

The published cytochrome *b* sequences for *A. sylvaticus* (*n* = 5: AF127543, AF127538, AF127537, AF127536 and ABO33695), *A. flavicollis* (*n* = 2: AJ311150 and AJ311151) and *A. mystacinus* (*n* = 2: AJ311146 and AJ311147) were downloaded from GenBank (see Table 1) and aligned to the new sequences using the ED editor [MUST package (Philippe 1993)].

The cytochrome *b* aligned sequences were analysed for saturation as described by Philippe & Douzery (1994) and Hassanin *et al*. (1998). Using the matrices of patristic and inferred substitutions calculated by PAUP 4 (Swofford 1998), the pairwise numbers of observed differences were plotted against the corresponding values for inferred substitutions (Philippe & Douzery 1994). The slope of the linear regression (S) was used to evaluate the level of saturation (Hassanin *et al*. 1998). When no saturation is observed in the data set, the slope equals one, whereas the slope tends towards zero as the level of saturation increases.

Analyses

The aligned sequences were analysed by distance [neighbour joining (NJ); Saitou & Nei 1987] and by maximum parsimony (MP; Fitch 1971) methods. We used PAUP 4.0b8 [NJ: minimum evolution (ME) criterion, Kimura's two parameter (K2P) distance estimator; MP: heuristic search, tree bisection reconnection (TBR) branch swapping option, Maxtrees = 1000 (Swofford 1998)]. The NJ analysis was performed assuming a gamma distribution for substitution rates across sites, where the parameter alpha (Yang 1996) and the proportion of invariant sites (*I*) were estimated with the maximum-likelihood method assuming the K2P phylogeny using PAUP 4.0b8. The robustness of inferences was assessed by bootstrap resampling (BP) (Felsenstein 1985) (1000 and 100 random repetitions for distance and MP analyses, respectively). A minimum spanning network was constructed using the MINSPNET algorithm available in the ARLEQUIN 2.0 program (Schneider *et al*. 2000). Nucleotide diversity, π, was estimated using the DNASP program (Rozas & Rozas 1997). A 'mismatch distribution' of substitutional differences between pairs of haplotypes was calculated within the main genetic lineages and compared with a fit to the Poisson model using DNASP (Rozas & Rozas 1997). We used a coalescent approach to validate the patterns of population growth or stability revealed by the mismatch distributions (Emerson *et al*. 2001). We used maximum likelihood to fit two models, assuming either that the population was stable through time, or that it grew (or declined) exponentially (Kuhner *et al*. 1995, 1998). Since both models are nested, they were compared with a likelihood-ratio test which follows a χ^2 distribution with one degree of freedom (there is an additional parameter in the latter model). This analysis was performed with the programs COALESCE and FLUCTUATE from the package LAMARC (http://evolution.genetics.washington.edu/lamarc.html). A model of DNA evolution with a transition/tranversion ratio equal to 2 was used with the empirical base frequencies (the results were not sensitive to this last parameter as they were similar if it is assumed that the base frequencies are equal to 0.25). The programs were run several times with different numbers of short and long Markov chains to check the consistency of the estimated parameters. The other parameters (θ with COALESCE, and θ and the population growth rate with FLUCTUATE) were allowed to vary, and were estimated at the maximum likelihood. The likelihood-ratio test was computed with twice the difference in the log-likelihoods provided by both programs: the null hypothesis being that the population was stable. This analysis was performed separately for the four subclades (1a, 1b, 2a and 2b).

The population genetic structure was determined by analysing the molecular variance and calculating ϕ_{ST} [analysis of molecular variance (AMOVA) available in ARLEQUIN 2.0; Schneider *et al*. 2000]. This method estimates the proportion of genetic variation at different hierarchical levels, using information from the geographical distribution of haplotypes and the pairwise distance between them. This analysis was performed at different hierarchical levels: among groups (corresponding to the observed subclades), among populations within each group (17 populations were defined according to geographical and ecological data) and within each population.

As populations of recent origin may not be at genetic equilibrium, the estimate of the timing of intraspecific divergence must be interpreted cautiously. Therefore, an approximate timing of divergence between the observed mitochondrial DNA (mtDNA) lineages was calculated on the basis of the percentage of genetic divergence obtained with a distance analysis (K2P estimator). As proposed by Edward (1997), the genetic distance between two different lineages was corrected for ancestral mtDNA polymorphism using the formula:

$$P_{net} = P_{AB} - 0.5(P_A + P_B)$$

Where P_{net} is the corrected distance between the isolated lineages A and B, P_{AB} is the mean genetic distance in pairwise comparisons of individuals from A vs. B, and P_A and P_B are mean genetic distance among individuals within these lineages. Two calibration points derived from palaeontological data were used for this analysis. First, the divergence time between *A. mystacinus* and all the 'small' *Sylvaemus* was estimated at approximately 7 Myr (Aguilar & Michaux 1996; Michaux *et al*. 1997) and second, the divergence between *A. sylvaticus* and *A. flavicollis* was estimated from fossil data to be approximately 4 Myr (Michaux & Pasquier 1974; J. Michaux, personal communication).

To identify whether there were differences in the rates of cytochrome *b* changes in different *A. sylvaticus* lineages or between studied specimens, relative-rate tests were conducted with each of them against the remaining lineages or individuals. The relative-rate tests were performed with RRTREE, version 1.0 (Robinson *et al*. 1998), which improves the test of Wu & Li (1985) taking into account taxonomic sampling and phylogenetic relationships. The NJ tree was chosen as the reference topology. *Apodemus flavicollis* was used as an outgroup. Relative-rate tests were performed on the proportions of synonymous (K_s) and nonsynonymous (K_a) substitutions.

Results

Haplotype variations and saturation analysis

We identified 99 haplotypes among the 102 *Apodemus sylvaticus* cytochrome *b* sequences (GenBank accession numbers AJ511877 to AJ511987). The complete data matrix comprised these 99 haplotypes as well as two *A. flavicollis* and two *A. mystacinus* sequences used as outgroups, chosen on the basis of a molecular phylogenetic study on the genus *Apodemus* (Michaux *et al*. 2002). This matrix provided 965 base pairs, of which 282 sites (29%) were variable and 226 (23%) were parsimony informative. The mean transition to transversion ratio was 2.29 and the nucleotide frequencies were 26.3%, 30.5%, 30.7% and 12.5% for C, T, A and G, respectively.

The saturation analysis showed that none of the events was affected by homoplasy with the exception of the transitions at the third codon position which showed a weak saturation ($S = 0.76$ and no plateau of saturation). However, as this substitution provided important phylogenetic information and as the use of weakly saturated events does not seems to interfere greatly in phylogenetic analyses (Yoder & Yang, 2000), all the events were included in the analyses.

Phylogenetic analyses

The distance analysis (Fig. 2) showed that the 99 *A. sylvaticus* haplotypes fell into two major clades: the first comprising the Italian, Balkan and Sicilian animals (clade 1), and the second corresponding to all the populations from North Africa and western, northern and central Europe (clade 2). These clades are well supported [bootstrap support (BP) of 97% and 88%, respectively] and separated by a high degree of genetic divergence (5.4% of K2P genetic distance). The first clade was divided into two subclades showing a large and robust differentiation (3.6% K2P distance; BP: 100%): a Sicilian one (subclade 1b) and an Italo-Balkan one (subclade 1a). A very well supported North African group (subclade 2a) (BP: 100%; 1.9% K2P distance) also appeared nested within the western European group (subclade 2b). No other geographical structure was observed with the exception of a weakly supported small subgroup (BP: 51/67) corresponding to Spanish and southern French animals (haplotypes 14–23). The MP analysis yielded one most parsimonious tree (*L* (*number of parsimony steps*) = 582 steps; *CI* (*consistency index*) = 0.53; *RI* (*retention index*) = 0.89) identical to the NJ tree. The bootstrap values resulting from this analysis are included in Fig. 2. The minimum spanning network (Fig. 3) shows a high differentiation between clades 1 and 2 (22 mutational steps). The Sicilian and North African populations also appeared well separated with 17 and nine mutational steps, respectively. Subclades 1a and 2a appeared as star-like topologies, suggesting a recent expansion from a small number of animals (Avise, 2000) whereas subclades 1b and 2b appeared more heterogeneous.

Nucleotide diversity and genetic structure

We calculated nucleotide diversity for each subclade. Moreover, to assess whether nucleotide diversity was higher within the potential refuge regions compared to the northern populations, subclade 2b was divided into two subgroups: the first corresponding to the Iberian and southern France populations and the second to all other European populations. The results of these analyses are summarized in Table 2 and indicate that the Sicilian animals have the highest level of nucleotide diversity.

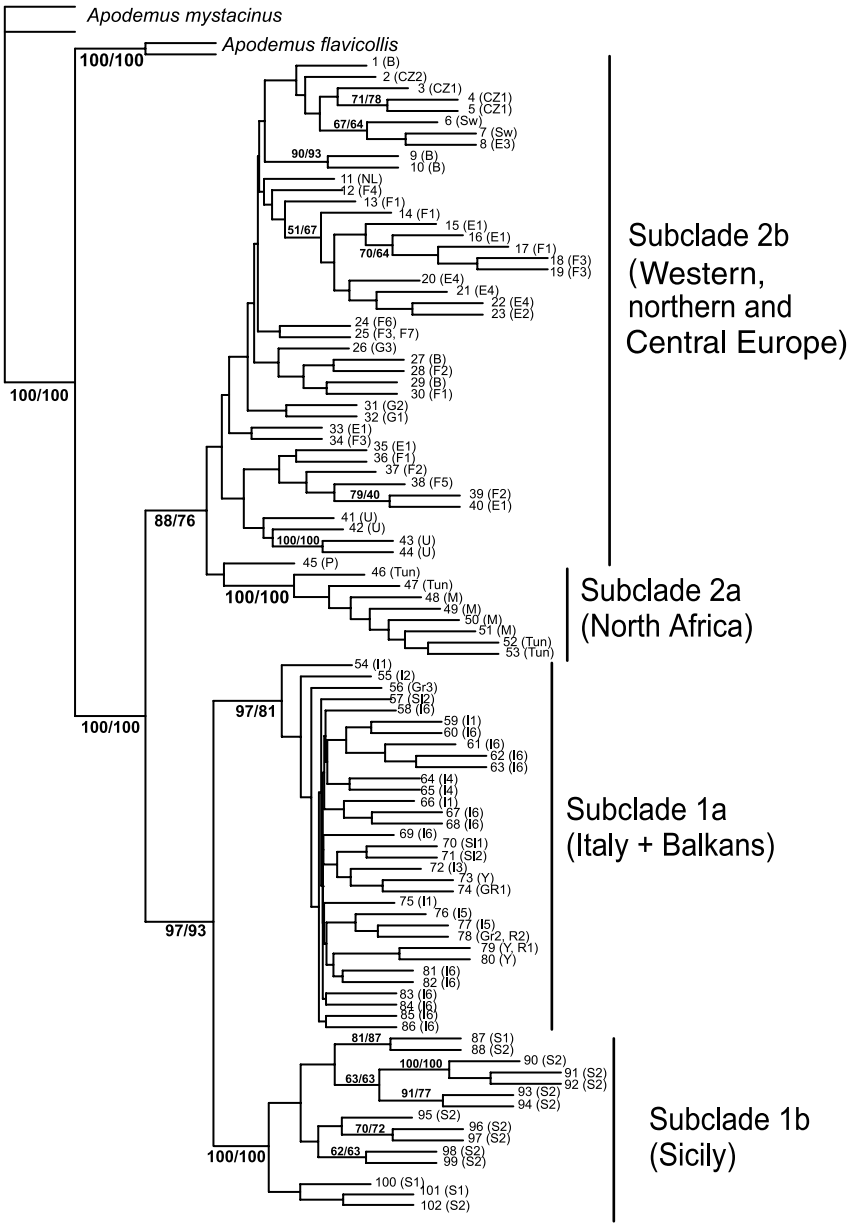

Fig. 2 Neighbour-joining tree for the 99 woodmouse mtDNA haplotypes (labelled from 1 to 99). Geographic origins (see Table 1 and Fig. 1) are shown in parentheses. Numbers indicated on the branches correspond to bootstrap support above 50% obtained in the NJ and MP analyses, respectively.

Within the continental populations, the southwestern group (subclade 2b1) was characterized by a significantly higher nucleotide diversity ($P < 0.05$) than either the northern group (2b2) or the other subclades. Among these (1a, 2a), the North African population showed a very low level of nucleotide diversity.

The AMOVA showed that the majority of the total mtDNA variation (76.2%) was distributed among the four genetic groups whereas a low percentage of this variation (3.8%) was observed among populations within the main lineages.

A signature of population growth (i.e. a bell-shaped distribution) was clearly evident in the distribution of pairwise distribution within the subclades 1a, 2a and 2b (Fig. 4). By contrast, a more heterogeneous distribution appeared in the Sicilian lineage (1b), suggesting a more stable population less subject to a recent expansion (Harpending *et al*. 1993). The coalescence analysis rejected the null hypothesis of a stable population for two subclades out of four: West Europe and North Africa. However, for Italy and Balkans (subclade 1a) the test was close to significance (Table 3). In all three cases the estimated population growth rate was positive, indicating an expansion for these subclades. For Sicily, there was no indication of population size change, and the null model was accepted. It is remarkable that the null hypothesis was rejected with the smallest sample size (North Africa), which suggests that the result for Sicily was not a result of lack of statistical power.

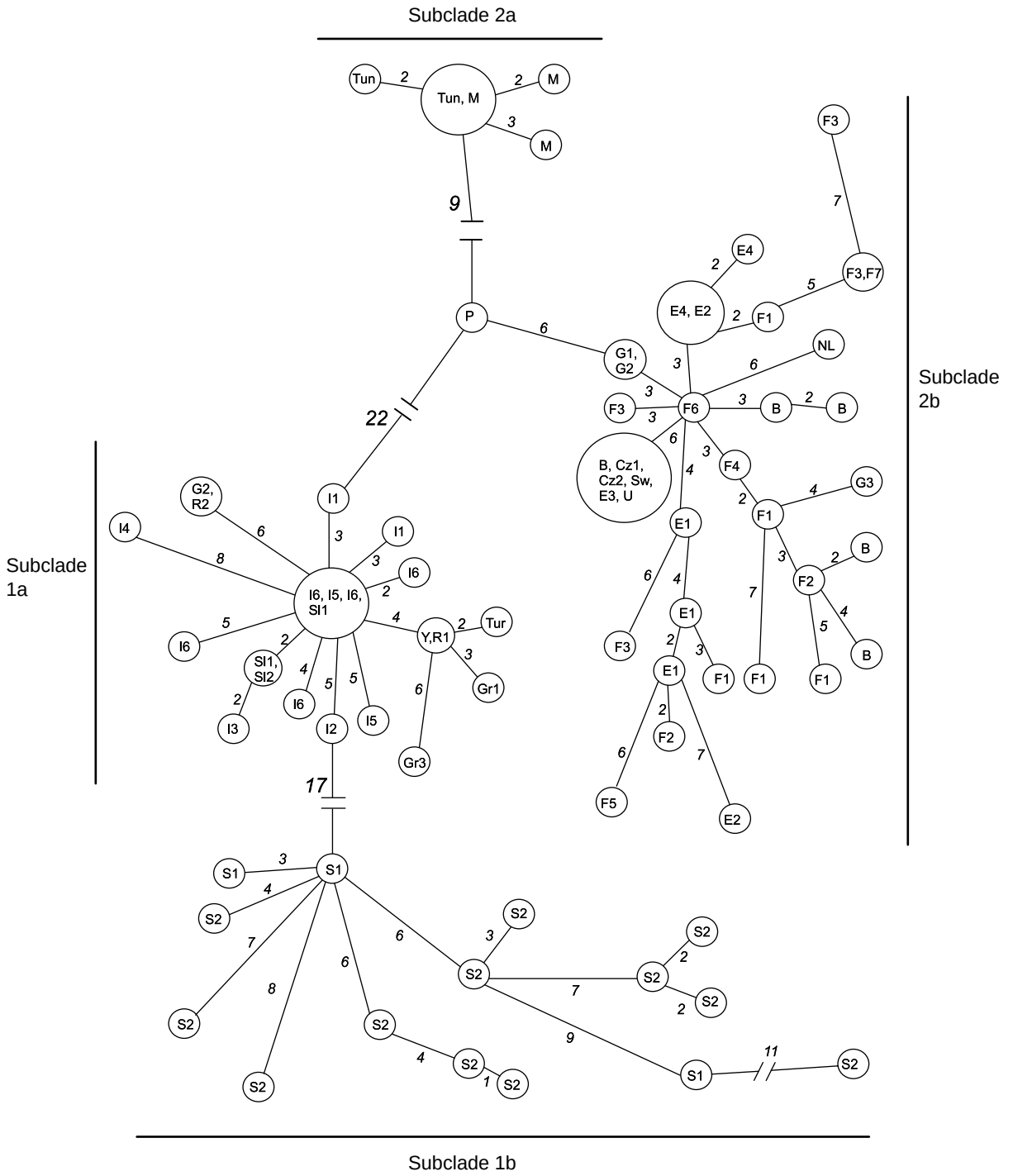

Fig. 3 A minimum spanning network constructed using mitochondrial cytochrome *b* gene sequences. Geographic origins (see Table 1 and Fig. 1) are indicated. For simplicity, haplotypes separated by only one mutational step have been associated. Numbers in italics correspond to the mutational steps observed between haplotypes.

Divergence time

The relative rate test indicated no significant rate of heterogeneity (both K_s and K_a) for cytochrome *b* between the different *A. sylvaticus* subclades and the *A. sylvaticus* samples.

The mean K2P distance between *A. mystacinus* and *A. flavicollis*/*A. sylvaticus* that diverged 7 Myr ago was 18.4%. The distance between *A. flavicollis* and all remaining *A. sylvaticus* that diverged 4 Myr ago was 11.4%. These values gave a rate of 2.6–2.85% K2P distance per million years

Table 2 Genetic variability observed within the four main genetic lineages of *Apodemus sylvaticus*

	Sample size	No. of haplotypes	Genetic divergence within each clade (% K2P distance)	Nucleotide diversity (P_i)	Standard deviation	Haplotype diversity (h)	Standard deviation
Clade 1							
Subclade 1a (Italy + Balkans)	26	25	1.22	0.0064	0.0008	0.0968	0.022
Subclade 1b (Sicily)	15	15	1.33	0.013	0.001	1	0.024
Clade 2							
Subclade 2a (North Africa)	8	8	0.29	0.0029	0.0005	1	0.063
Subclade 2b1* (southern Europe)	27	23	1.41	0.0087	0.0007	0.981	0.023
Subclade 2b2* (northern Europe)	26	20	1.09	0.0052	0.001	0.965	0.028

*The subclade 2b is divided into two different subgroups corresponding to the southern and northern European populations, respectively.

Table 3 Results of the coalescence analysis on the four main genetic lineages of *Apodemus sylvaticus*

	Log-likelihood			
Sub-clades	stable population model	exponential change model	χ^2	P
Italy, Balkans (1a)	–0.0028	1.6249	3.26	0.071
Sicily (1b)	0.0265	0.1142	0.18	0.675
North Africa (2a)	0.0035	2.2495	4.49	0.034
West Europe (2b)	–0.1132	11.2927	22.81	0.0001

(*mystacinus/flavicollis–sylvaticus* and *flavicollis/sylvaticus*). When this rate was applied to the different dichotomies within *A. sylvaticus* and taking into account the correction for ancestral mtDNA polymorphism, the following molecular datings were obtained: 1.5–1.6 Myr for the separation between clades 1 and 2, 0.8–0.9 Myr for the separation between clades 1a and 1b (see Table 2) and 0.4 Myr for the separation of the North African population.

Discussion

Association of geographical structure and historical factors

The distance, maximum parsimony and network analyses clearly show that *Apodemus sylvaticus* populations are separated into four main genetic lineages that have nonoverlapping geographical distributions. These results are in agreement with those obtained on the basis of random fragment length polymorphism (RFLP) methods (Michaux *et al*. 1996a,b, 1998a,b; Libois *et al*. 2001). This geographical structure is highly supported as 76.2% of the total mtDNA variation (AMOVA analysis, Table 3) is distributed among the four genetic groups. In contrast, as already observed on the phylogenetic trees, a smaller variability exists among populations within each of these lineages (3.8% of total mtDNA variation).

Continental European populations are separated into two main lineages: the first one is widespread from southern Spain in the south to Sweden in the north and the Ukraine in the east; the second lineage is limited to Italy, the Balkans and the western part of Turkey. This strong geographical structure corresponds to the first category of phylogeographic model defined by Avise (2000). As already proposed for several other European species (Taberlet *et al*. 1998; Hewitt 2001; Seddon *et al*. 2001), this structure could be explained by isolation of two woodmouse groups in two different refugia (Iberian peninsula and Italy/Balkans) during one of the previous ice ages. This isolation was probably reinforced by the presence of the Alps which played, and probably continue to play, the role of a biogeographic barrier for the woodmouse (Michaux *et al*. 1996b). These two factors probably prevented genetic exchanges between both groups for a long time and led to the strong genetic differences observed within this species. In contrast, the frequent drops in the level of the Adriatic Sea during the Quaternary ice periods (Strahler & Strahler 1989) probably allowed the Italian and Balkan woodmouse populations to remain in contact. Likewise, the population of woodmice found in western Turkey is very similar to that from the Balkans, suggesting recent contacts between them, probably during one of the last ice ages when the Marmara Sea was replaced by dry lands.

Do these lineages correspond to genetic groups within A. sylvaticus *or to different species?*

Johns & Avise (1998) stated that cytochrome *b* differentiation was highly congruent with traditional species boundaries. More recently, Bradley & Baker (2001) used a partition of genetic distances values (using the K2P) in determining specific boundaries under the Genetic Species

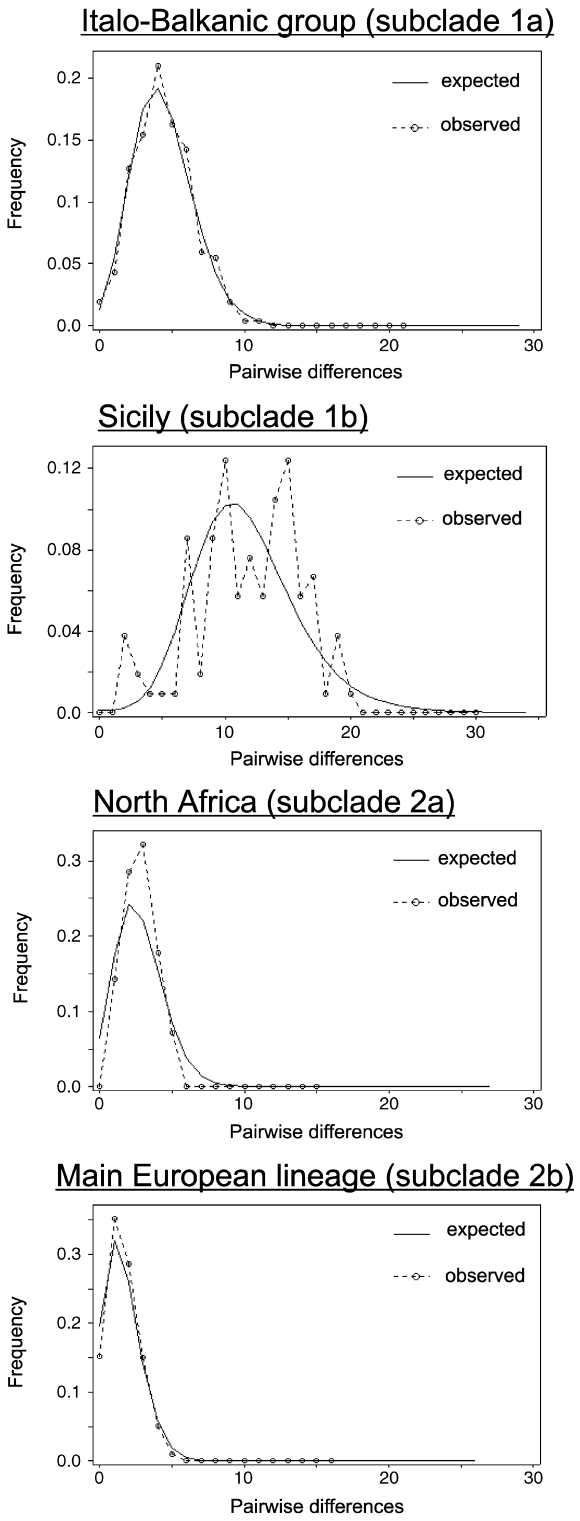

Fig. 4 Mismatch distribution for mtDNA types from the four major *Apodemus sylvaticus* genetic lineages. The expected frequency is based on a population growth-decline model (for the Italo-Balkan, Sicilian, North African and main European groups, respectively: θ initial = 0, 2.4, 0 and 0, θ final = 1000, τ = 4.4, 9.4, 2.7 and 1.6), determined using the DNASP v3.5 program (Rozas & Rozas 1997).

Concept. Taking this concept into account as well as previous data on rodents (Bradley & Baker 2001), it seems that the level of genetic divergence between the different lineages of *A. sylvaticus* (maximum: 5.4% K2P estimator) corresponds to different intraspecific genetic groups or subspecies. Indeed, the level of genetic divergence between *A. sylvaticus* and other closely related and well-defined species, such as *A. flavicollis* or *A. alpicola*, reaches at least 10–12% (Michaux *et al*. 2002).

The divergence time estimated on the basis of two palaeontological calibration points, indicates a separation time of 1.5–1.6 Myr between the ancestral haplotypes which led to the two European lineages. Therefore, it appears that the intraspecific structure within *A. sylvaticus* developed during the Quaternary. This result is consistent with the hypotheses of Avise (2000), Seddon *et al*. (2001) and Hewitt (2001).

Refuge regions and postglacial recolonizations

As predicted by the expansion/contraction model (Nichols & Hewitt 1994; Ibrahim *et al*. 1996; Santucci *et al*. 1998) the analysis of nucleotide diversity (Table 2) confirms that southwestern Europe (Iberian peninsula and southern France) was a refuge region for *A. sylvaticus*. Indeed, these populations are characterized by a genetic diversity significantly higher ($P < 0.05$) than in northern populations. Therefore, it is probable that woodmice recolonized and expanded in the main part of the Western Palearctic region from southwestern Europe at the end of the last ice age. This scenario of expansion is corroborated by a bell-shaped distribution in the mismatch distribution analysis for the main European lineage (Luikart *et al*. 2001). Thus, the Pyrenees were not an effective barrier to the northward expansion of the woodmouse (Michaux *et al*. 1998b). This does not confirm the assertion of Bilton *et al*. (1998) proposing that the widespread small mammal species did not recolonize northern Europe from the Mediterranean peninsulas. However, their hypothesis is not strongly supported by appropriate sampling ($n = 1$ for each peninsula).

In contrast to the southwestern group, the Italo-Balkan populations are characterized by a lower genetic diversity. This observation is somewhat surprising as palaeontological (Aguilar and Michaux, unpublished data) and palaeoclimatological (Reille & de Beaulieu 1995; Tzedakis *et al*. 1997) data attest that Italy and the Balkans were a refuge region for *A. sylvaticus* during the Quaternary glaciations. This low genetic variability could be explained by a genetic bottleneck which appeared during one of the last ice ages. This hypothesis is corroborated by three results: the very short branch lengths between haplotypes within this group in the distance analysis; the star-like topology in the minimum spanning network suggesting a rapid expansion

from a small number of founder animals and a low value of haplotype (*h*) and nucleotide (*Pi*) diversity (Table 2) (Avise, 2000). The important decrease in population size could have resulted from different environmental factors:

(i) Palaeoclimatological data (Tzedakis 1994) indicate that a great part of the Balkans was more arid than the other western European regions during the early Quaternary glaciations. This climate probably influenced the distribution of the woodmouse habitat (woodlands) leading to a fragmentation of populations.

(ii) The number of closely related species of *Apodemus* (*A. flavicollis*, *A. uralensis*, *A. agrarius*, *A. mystacinus*) is higher in Central Europe and the Balkans compared to western Europe (*A. flavicollis* and *A. alpicola* only in the Alps) (Schilling *et al*. 1986; Wilson & Reeder 1993). Therefore, as these species often live in the same habitats, interspecific competition may have played a role in the decrease in population size and therefore of genetic variability of the woodmouse.

A genetic bottleneck could also explain why the Italo-Balkan populations did not contribute to the postglacial colonization of the northern regions, as expansion could only have occurred after population growth. During this time, the western group rapidly expanded towards northern and central Europe, thereby preventing a northern colonization by the Italo-Balkan animals. Indeed, once established, resident rodents often aggressively exclude newcomers (Granjon & Cheylan 1989). Moreover, the presence of topographic obstacles such as the Alps and the Carpathian mountains might slow down a postglacial expansion of the Italo-Balkan group. This is the first report of such a pattern of postglacial colonization (Taberlet *et al*. 1998; Hewitt 1999, 2001).

The 'Sicilian enigma'

Until now, the origin of the Sicilian woodmouse population was unknown (Michaux *et al*. 1998). Our phylogenetic analyses indicate that they are related to the Italo-Balkan lineage. However, in contrast to the other western Mediterranean islands where the woodmouse was introduced recently and is genetically similar to continental populations (Michaux *et al*. 1996a,b, 1998b), the Sicilian lineage is very differentiated from the Italian ones (3.2% K2P distance). Their separation is estimated to have occurred 0.8/0.9 Myr ago. The results, obtained with RFLP methods (Michaux *et al*. 1998), confirm this observation. Moreover, the mismatch distribution and the coalescent analyses show that this population was not subjected to a recent expansion but rather appears more stable than all the other European woodmouse populations (Harpending *et al*. 1993). A high value of *h* and *Pi* corroborate this interpretation (Avise, 2000).

Two hypotheses can explain this:

(i) The Sicilian population has been isolated from the continent for at least 0.8 Myr allowing ancestral haplotypes to survive and diverge whereas woodmice disappeared from continental regions for several reasons (i.e. genetic bottlenecks associated with more severe climate changes or interspecific competition). However, this hypothesis is at odds with the palaeontological and geological data which attest the presence of *A. sylvaticus* in Sicily for only 70 000–50 000 years (M. Sara, personal communication) and the existence of several connections between Sicily and Italy for the last million years (Thaler 1973; J. J. Jaeger, personal communication).

(ii) An old lineage could have survived elsewhere in southern Italy and entered Sicily during the last glaciation, 70 000 years ago. This ancient stock would then have remained trapped on the island until present times where it suffered less from the last ice age climate, whereas the continental population was replaced by a modern stock spreading from other Italo-Balkan refuges during the postglacial era.

Whatever the hypothesis explaining the genetic differentiation of this population, Sicily appears as a 'hot spot' of genetic diversity for the woodmouse. This has also been observed for the bird *Alectoris graeca* (Lucchini & Randi 1998).

Colonization of northern Africa

As already proposed by Libois *et al*. (2001) on the basis of RFLP methods, the phylogenetic analyses performed here show that the North African woodmice are clustered in a specific subclade (100% BP) itself nested within the main European group (clade 2). This observation strongly suggests that the North African woodmice may have a southwestern European origin. This is supported by the absence of genetic affinities between the North African woodmice and either the Sicilian, Italian, or Balkan populations. The most closely related European haplotype (45) was found in the central part of Portugal, suggesting that their introduction probably occurred via the Strait of Gibraltar. The high value of *h* and low value of *Pi* (Table 2), the mismatch distribution [bell-shape distribution (Fig. 4)], the coalescent results and the star-like topology observed in the minimum spanning network (Fig. 3) all suggest that the genetic structure of these woodmice is the consequence of the very recent introduction of a small number of animals followed by rapid population growth (Avise, 2000; Luikart *et al*. 2001). However, the divergence time analysis proposes a separation time (0.4 Myr: middle Pleistocene) between the European and North African population.

This estimate is similar to that obtained on the basis of RFLP studies (Libois *et al*. 2001), but is at odds with the palaeontological data which suggest a recent (10 000 years: Holocene) colonization of the woodmouse in North Africa (Jaeger 1975; J. Jaeger, personal communication 1999; Burgio 1998) as well as the overall low genetic variability characterizing the North African woodmouse. This can be explained by two hypotheses:

(i) A recent colonization event involving only a few individuals may have occurred as a result of anthropogenic introductions (Michaux *et al*. 1996b, 1998; Dobson & Wright, 2000). In this case, the relatively high level of genetic divergence between European and North African woodmice may be a result of the presence of divergent haplotypes in the introduced founder individuals. This is compatible with the high diversity of mitochondrial types observed in the Iberian region (Table 2).

(ii) A small initial 'inoculation' occurred during the middle Pleistocene period and only recently expanded. Nevertheless, it is difficult to explain why this founder population would not have invaded other North African regions during 400 000 years, considering that the ecoclimatic characteristics of these regions were favourable for woodmice during the Pleistocene era (Jaeger 1975; Blondel 1995). Moreover, this hypothesis does not agree with the palaeontological data (Jaeger 1975).

In conclusion, we suggest that the woodmouse in North Africa originated from southwestern Europe and is probably the result of a recent anthropogenic introduction as has been suggested by Dobson (1998) and Dobson and Wright (2000).

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