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Taxonomy, evolutionary history and biogeography of the broad-toothed field mouse (*Apodemus mystacinus*) in the eastern Mediterranean area based on mitochondrial and nuclear genes

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The broad-toothed field mouse (*Apodemus mystacinus*) is distributed throughout the Balkan Peninsula, Asia Minor and the Middle East. It is generally split into two different specific entities: *Apodemus epimelas* occurs on the Balkan Peninsula and *A. mystacinus* inhabits Asia Minor and the Middle East. This analysis, based on two mitochondrial regions (cytochrome *b* and the D-loop) and the *interstitial retinol binding protein (IRBP)* nuclear gene, confirms an important level of genetic divergence between the animals from these regions and their separation from each other at least 4.2–5.1 Mya, which is in favour of a distinct specific status. Finally, the broad-toothed field mice from south-western Turkey appear to be closely related to the animals from Crete but highly distinct from the populations of the other Oriental regions. This supports a distinct subspecific level (*A. m. rhodius*) for the insular animals and also for those from south-western Turkey. From a biogeographical point of view, it can be assumed that either late Pliocene or early Pleistocene cooling led to the isolation of two main groups of *A. mystacinus*, one in the Balkan region and the other one in Turkey and the Near East (Syria and Israel). In this region, it is suggested that a more recent event appeared during the Quaternary period, isolating broad-toothed field mice in Crete and leading to the appearance of two well-differentiated genetic groups: one in Crete and south-western Turkey, and the other widespread in northern and eastern Turkey as well as in the Near East.

ADDITIONAL KEYWORDS: eastern Mediterranean region – *IRBP* nuclear gene – mitochondrial control region – mitochondrial *cytochrome b* gene.

INTRODUCTION

The broad-toothed field mouse, *Apodemus mystacinus* (Danford & Alston, 1877), is a small rodent of the Murinae subfamily distributed throughout the eastern Mediterranean region. It lives in rocky and stony habitats in various environments such as cultivated areas, woodlands or ruins. It is clearly distinguishable from the other European *Apodemus* species by different morphological characters such as a greater body size and a dark-grey coloration of the fur. Its range extends from the Balkan region (Albania, the former Yugoslavia, Bulgaria and Greece) (Mitchell-Jones *et al*., 1999) to the Near East (Turkey, Georgia, Jordan, Lebanon, Israel and Iraq) (Kock, Malec & Storch, 1972). It is also present in Corfu, Rhodes, Karphatos and Crete, as well as in several Aegean islands (Niethammer, 1978).

The taxonomic status of this species is still unclear. Two subspecies were firstly described within *A. mystacinus*: *A. m. epimelas* (Nehring, 1902) occurring on the Balkan peninsula (Terra typica: Parnas

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Mountains, Greece) and *A. m. mystacinus* (Danford & Alston, 1877) inhabiting Asia Minor and the Middle east (Terra typica: Bolkar Daglari Mountains, Asia Minor). This classification is supported by Spitzenberger (1973) on the basis of morphological studies. Another subspecies has also been described in the islands of Crete, Karpathos and Rhodes, namely *A. m. rhodius* (Ondrias, 1966).

On the basis of palaeontological data, however, Storch (1977) considers that *A. epimelas* and *A. mystacinus* are two distinct species, an opinion also shared by Mezhzherin (1997), in his systematic revision of the *Apodemus* species of northern Eurasia.

To date only a single genetic study based on allozyme data (Filippucci, Macholán & Michaux, 2002) has been performed on this species and tends to confirm a specific status for *A. epimelas* and *A. mystacinus*. Nevertheless, a new molecular study based on DNA sequences and carried out on different specimens from European and oriental regions would be extremely useful to highlight the taxonomic debate.

From a biogeographical point of view, the existence of these various taxa raises many interesting questions:

1. How old are the morphologic differences between the European and oriental populations? Are they resulting from an ancient allopatric isolation associated to a previous geological phenomenon or climate change (e.g. the Quaternary ice ages)?
2. As the Marmara Sea was frequently replaced by dry lands during the Quaternary ice ages, why is the European form absent from Turkey and vice-versa?
3. As suggested by the description of several subspecies within the oriental group (Spitzenberger, 1973), is it possible to distinguish different genetic lineages in this region. How and when did they eventually appear?
4. What is the origin of the *A. mystacinus* populations in Crete? As *Apodemus sylvaticus* (J. Michaux, unpubl. data), did it invade this island from Greece, or did it come from oriental regions, similar to another rodent, *Acomys minous* (Barome *et al*., 2001)?
5. When did it invade the oriental Mediterranean islands? Is it, like *A. sylvaticus* (J. Michaux, unpubl. data) and *Acomys minous* (Barome *et al*., 2001), a recent colonist associated with anthropogenic introductions?

The aims of this study are to address these questions and to better determine the taxonomic status of *A. mystacinus.* Several well-distributed populations are analysed using sequences of the nuclear *IRBP* gene and two mitochondrial regions, the *cytochrome b* gene and the D-loop region.

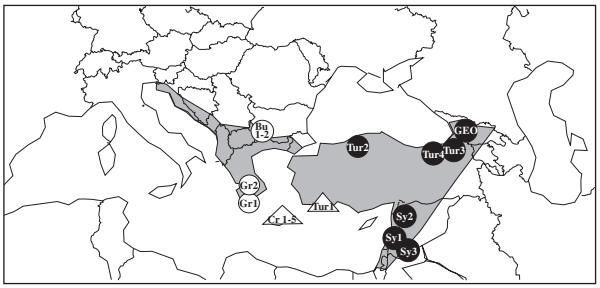

Figure 1. Geographic distribution of the *Apodemus mystacinus* samples. The shaded zones correspond to the distribution area of this species (as described by Mitchell-Jones *et al*., 1999 and Kock *et al*., 1972). White circles, black circles and triangles correspond to clades A, B1 and B2, respectively. See Table 1 for sample symbols.

MATERIAL AND METHODS

Biological material

A total of 21 *Apodemus mystacinus* from 15 localities widespread throughout a large part of the species distribution area were analysed (Table 1, Fig. 1). One *Apodemus agrarius* and one *Apodemus peninsulae* were also analysed to sequence their D-loop region.

Laboratory methods

DNA was extracted from ethanol-preserved tissue following Sambrook, Fritsch & Maniatis (1989) or Bahl & Pfenninger (1996). Tissues were taken from the *Apodemus* tissue collection of J. R. Michaux (JRM numbers) and those of E. Bellinvia (EB numbers).

A large portion of the *cytochrome b* gene (972 bp) was amplified using the universal PCR primers L7 (5′-ACCAATGACATGAAAAATCATCGTT-3′) and H16 (5′-ACATGAATYGGAGGYCAACCWG-3′) (Kocher *et al*., 1989). Moreover, 782 bp of the *IRBP* gene were amplified using the PCR primers I1 and J2 (Stanhope *et al*., 1992). Finally, the whole D-loop and flanking tRNA genes (about 1000 bp) were amplified in two overlapping segments of about 600 bp each. Primers 1 (5′-ATAAACATTACTCTGGTCTTGTAAAC-3′) and 2 bi (5′-CACAGTTATGGAAGTCTTGG-3′) were used to obtain a PCR product including the whole *tRNAThr* and *tRNAPro* genes and about 460 bp of the D-loop up to about half of the central domain. The second segment, extending from about one half of the D-loop central domain to the beginning of the 12S tRNA region, including the *tRNAPhe* gene, was produced with primers 3 (5′-CGTTCCCCTAAATAAGACA-3′) and 4 (5′-TAATTATAAGGCCAGGACCA-3′).

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

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

Geographic origin	Tissue	Sample symbols (see Fig. 1)	Accession numbers (*IRBP* gene)	(*cytochrome b* gene)	(D-loop)
Apodemus mystacinus					
Greece, Peloponissos, Taygetos	JRM-480	Gr 1	AJ48222	AJ48228	AY623070
Greece, Peloponissos, Delphes	JRM-281, JRM-282	Gr 2	AJ48222, AJ48224	AJ48229, AJ48230	AY623069, AY623064
Crete	EB-1816	Cr 1	AJ48211	AJ48232	AY623071
Crete, Anogia	JRM-458	Cr 2	AJ48213	AJ48235	AY623073
Crete, Mamas	JRM-464	Cr 3	AJ48214	AJ48234	AY623072
Crete, Nida plateau	JRM-477, JRM-478	Cr 4	AJ48212, AJ48215	AJ48231, AJ48233	AY623074, AY623075
Bulgaria, Ploski, Blagoevogard	JRM-535	Bu 1	AJ48223	AJ48227	AY623063
Bulgaria, Breznica	EB-177	Bu 2	–	–	AY623068
Syria, Bloudan	JRM-542	Sy 1	AJ48220	AJ48236	AY623065
Syria, Bloudan	JRM-543	Sy 1	–	–	AY623078
Syria, Slinfeh	JRM-544	Sy 2	AJ48219	AJ48237	AY623080
Syria, Quanawat	JRM-261	Sy 3	AJ48216	AJ48238	AY623077
Syria, Quanawat	JRM-536	Sy 3	–	–	AY623081
Georgia	JRM-545	Geo	AJ48218	AJ48240	AY623083
Turkey, Antalya	EB-1169, EB 1170	Tur 1	AJ48210, AJ48209	AJ48226, AJ48225	AY623076, AY623067
Turkey, Safran Bolou	EB-178	Tur 2	AJ48208	–	AY623079
Turkey, Damar	JRM-262	Tur 3	AJ48217	AJ48239	AY623066
Turkey, Damar	EB-98	Tur 4	–	–	AY623082
Apodemus agrarius					
GenBank/CZ- Krásná Lipa			Suzuki *et al*., 2003	Suzuki *et al*., 2003	AY588250
Apodemus peninsulae					
GenBank/Bajkal, Russia			Suzuki *et al*., 2003	Suzuki *et al*., 2003	AY588251
Mus musculus					
GenBank			Suzuki *et al*., 2000	Bibb *et al*. (1981)	
Rattus norvegicus					
GenBank			Serizawa, Suzuki & Tsuchiya (2000)	Gadaleta *et al*. (1989)	

buffer, 10 μL of purified water and 0.2 μL of 5 U μL^{-1} Promega *Taq* DNA polymerase. Approximately 200 ng of DNA extract were used per PCR amplification. Amplifications were carried out in a Labover PTC100 thermal cycler using 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 and run on an ABI 310 (Applied Biosystems) automated sequencer.

Sequence alignment and saturation analysis

Published sequences for *cytochrome b* and *IRBP* of *A. agrarius*, *A. peninsulae*, *Mus musculus* and *Rattus norvegicus* were downloaded from GenBank (Table 1) and aligned to our new sequences using the ED editor (MUST package; Philippe, 1993). The program AFAS (MUST package; Philippe, 1993) was used to combine the aligned matrices of IRBP, D-loop and cytochrome *b*.

Following Philippe & Douzery (1994) and Hassanin, Lecointre & Tillier (1998), we examined the IRBP, D-loop and cytochrome *b* data sets for saturation. Using the matrices of patristic and inferred substitutions calculated by PAUP v. 4b8 (Swofford, 2000), the pairwise numbers of observed differences was 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.

Phylogenetic reconstructions

Firstly, each gene was studied separately, then the combined matrix for *IRBP* (736 bp), *cytochrome b* (976 bp) and D-loop (1063 bp) sequences was used to determine more precisely the phylogenetic position of the different *A. mystacinus* lineages.

Before combining these different genes into single matrices, the level of incongruence between genes was tested using PAUP v. 4b5 (option Hompart). This approach uses the incongruence length difference (ILD) test with the parsimony criterion; 1000 randomizations were performed on variable sites only (Farris, 1985).

After alignment of the sequences, the general time reversible model (GTR) and the Kimura's 2 parameters (K2P) estimator were used for the calculation of genetic distances. The GTR analyses were 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 GTR phylogeny using PAUP v. 4.0b8. Maximum parsimony (MP; heuristic search, tree bisection reconnection (TBR) branch swapping option) and maximum likelihood (ML; GTR model of sequence evolution) analyses were also conducted using PAUP 4.0b8 (Swofford, 2000).

The robustness of inferences was assessed by bootstrap resampling (BP) using 1000 random repetitions for MP and distance analyses, and 100 for ML.

Divergence time

Firstly, to identify whether there are differences in rates of *cytochrome b*, D-loop and *IRBP* changes between the different *A. mystacinus* lineages, relative rate tests were conducted with each lineage against the remaining ones. The relative-rate tests were done with RRTree, version 1.0 (Robinson *et al*., 1998) which improves the test of Wu & Li (1985) by taking into account taxonomic sampling and phylogenetic relationships. The three DNA regions were analysed separately. The ML tree for each region was chosen as the reference topology and *A. peninsulae* and *A. agrarius* were used as outgroups. For non-coding regions (D-loop), relative-rate tests were performed on the proportion of all the substitutions types (K). For coding sequences (*cytochrome b*), relative-rate tests were performed on the proportions of synonymous (K_s) and non-synonymous (K_a) substitutions.

Secondly, to apply a molecular clock and to estimate times of divergence, we estimated the ML (the search was constrained to clock-like evolution) tree based on the *cytochrome b* data set with *M. musculus* and *R. norvegicus* as outgroups. The D-loop and IRBP data matrices were not used for this analysis as explained in the Results. The inferred maximum likelihood distances were used to estimate separation times (Michaux *et al*., 2001). Two calibration points derived from palaeontological data were used. Firstly, the *Mus*/*Rattus* dichotomy at 12 Mya (Jaeger, Tong & Denys, 1986; Jacobs, Winkler & Murry 1989, Jacobs *et al*., 1990; Jacobs & Downs, 1994) and secondly, the divergence time between the *Apodemus* and *Sylvaemus* subgenera estimated at 7.9 Myr (Michaux *et al*., 1997, 2002).

RESULTS

New sequences

All of the sequences generated in this study were deposited in GenBank under accession numbers AY623063 to AY623083, AY588250 and AY588251 (D-loop), AJ748225 to AJ748240 (*cytochrome b*) and AJ748208 to AJ748224 (*IRBP*) (Table 1).

For methodological reasons, 17 *A. mystacinus* sequences were obtained for *IRBP*, 16 for *cytochrome b* and 21 for the D-loop. The D-loop of one *A. agrarius* and one *A. peninsulae* was also sequenced.

The alignment of the *IRBP* sequences of 19 individuals comprises 736 nucleotides of which 71 (9.6%) are variable and 25 (3.4%) are parsimony informative. No heterozygotic sites were found on the sequences studied. The average ratio of transitions to transversions (TS/TV) is 2.39, ranging from 1.2 to 5.1. The alignment of the *cytochrome b* gene consists of 976 nucleotides for 18 specimens, 266 (27%) of which are variable and 215 (22%) parsimony informative. The average ratio of TS/TV is 1.79, ranging from 0.80 to 5.34. The complete alignment of the D-loop mitochondrial region comprises 1063 sites for 23 individuals. Of these, 272 (25.6%) are variable and 173 (16.3%) are parsimony informative. The average ratio of TS/TV is 1.56, ranging from 0.8 to 6.7. The concatenated sequence data matrix for the 16 *A. mystacinus* and the two outgroups comprises 2775 nucleotide sites, 606 (21.8%) variable and 368 (13.3%) parsimony informative.

Saturation analysis

Saturation analysis of the *IRBP* and *cytochrome b* data set indicates that there is no saturation for transitions and transversions at the three-codon positions. Therefore we included all events at the three-codon positions of these two genes for all phylogenetic analyses. Saturation analysis of the D-loop data showed that transversions are moderately affected by homoplasy. On the contrary, transitions are more saturated ($S = 0.45$). However, as exclusion of transitions leads to an important lack of information, they were retained in further analyses.

Phylogenetic relationships between the *Apodemus mystacinus* lineages

(1) Independent analysis of the three genetic regions

Whatever the genetic region, it can be seen (Fig. 2) that the specimens are split into two main clades. The first (clade A) corresponds to the Balkan animals, while the second represents the populations from Turkey, Syria, Georgia and Crete. These clades are very well supported for the D-loop and the *cytochrome b* gene (BP: 99–100%) but they are less well supported for the *IRBP* gene (BP: 49–87%).

Within the second clade, two subclades are also distinct. Subclade B1 corresponds to the animals from north-western and eastern Turkey, Syria and Georgia; subclade B2 corresponds to the animals from Crete and south-western Turkey (the exception being for *IRBP*, which only corresponds to the specimens from Crete). These two groups are strongly supported for the *cytochrome b* gene (BP: 85–98%). On the contrary, the robustness of subclade B1 is lower for the D-loop and *IRBP* (BP: 36–62%).

(2) Combined analysis of the three genetic regions

Notwithstanding minor discrepancies between the branching patterns obtained from the D-loop and *cytochrome b* data sets, the ILD test showed no significant incongruence between the two mitochondrial markers ($P = 0.15$). Despite significant incongruences found when all three regions are combined ($P = 0.001$), they were concatenated for the 16 *A. mystacinus*, one *A. agrarius* and one *A. peninsulae* samples sequenced for all the three markers. Therefore, more sites were available for analysis.

A consensus tree, constructed from the topologies retrieved by MP, ML and neighbour-joining, is presented in Fig. 3. Again, the two main clades (A and B) are strongly supported (BP: 100%). Moreover, the two subclades B1 and B2 appear much more robust (BP: 67–100%). Within them, the animals from south-western Turkey are associated with the population from Crete.

A summary of the K2P sequence divergence values is presented in Table 2, both within and between the observed clades. To determine the precise genetic relationships between the animals from Crete and those from south-western Turkey, subclade B2 was divided into two parts. The *cytochrome b*, D-loop and *IRBP* sequences indicate that the Balkan populations are strongly separated from the oriental animals with an important level of genetic divergence (means of 13.3, 10.7 and 1.7% of K2P distance, respectively). Within this last group, the two mitochondrial regions show that the animals from south-western Turkey are separated from those from other oriental regions (2.9 and 1.7%, respectively). On the contrary, they are very similar to those from Crete (0.9 and 0.85%, respectively). The *IRBP* results are less clear and show a closer relationship between all the Turkish populations compared to the animals from Crete.

Divergence time

The relative rate test (Robinson *et al.*, 1998) indicated no significant rate heterogeneity (both K_s and K_a) for the cytochrome *b* and D-loop regions between the different clades. On the contrary, K_s comparisons for *IRBP* showed marked differences in evolutionary rates, clade A exhibiting an elevated rate as compared to the other clades. This excluded the use of *IRBP* data for divergence time estimates as the molecular clock is not homogeneous for it. Moreover, the use of the D-loop was also impossible for divergence time estimates

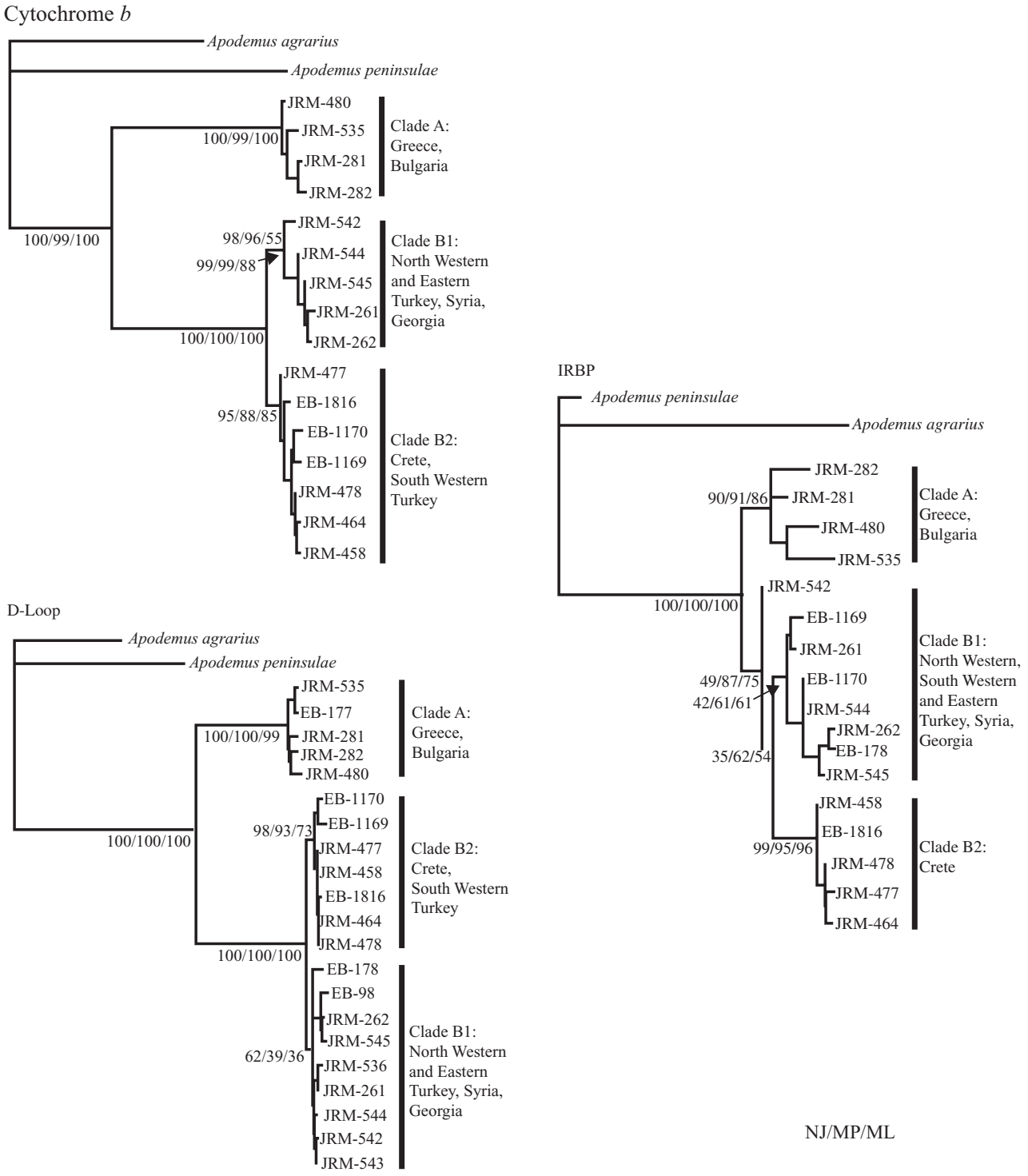

Figure 2. Neighbour-joining tree obtained for the three studied genetic regions, the mitochondrial *cytochrome b* and D-loop regions, and the nuclear *IRBP* gene. *Apodemus agrarius* and *A. peninsulae* are used to root the tree. For each main node, the different robustness indices are indicated as follows: distance bootstrap support/maximum parsimony bootstrap support/maximum likelihood bootstrap support.

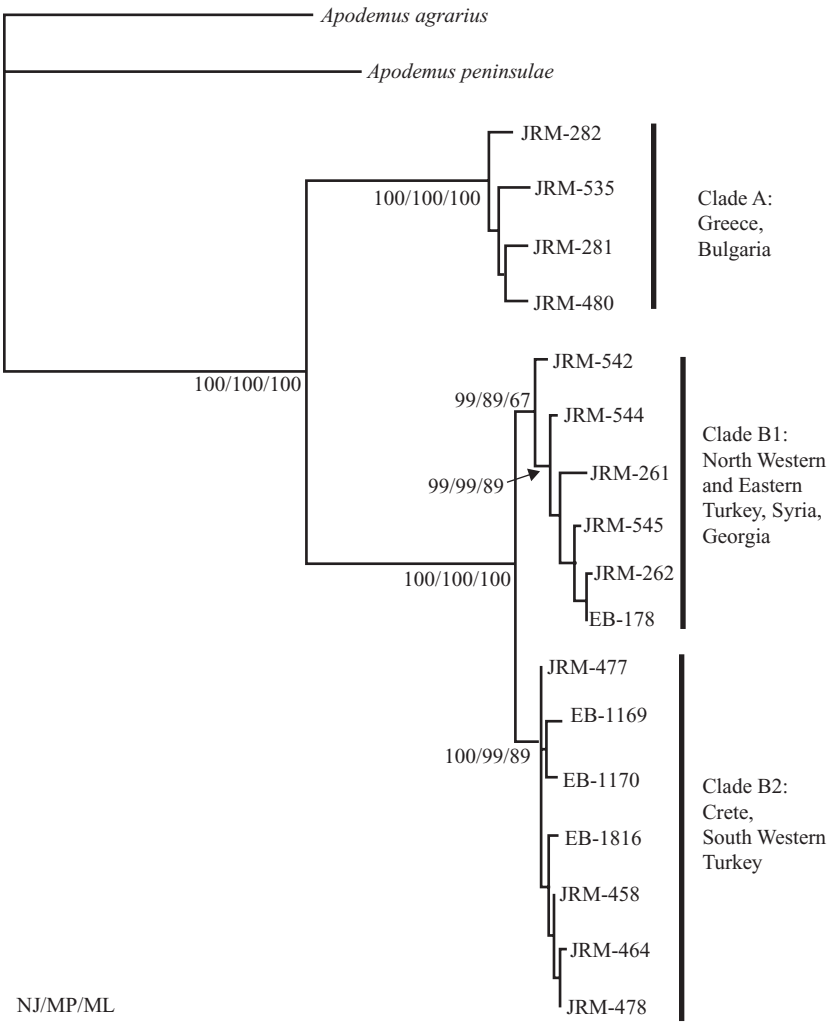

Figure 3. Neighbour-joining tree obtained from the analysis of the concatenation of the three genetic regions for 16 *Apodemus mystacinus* individuals and 2775 positions. *A. agrarius* and *A. peninsulae* are used to root the tree. For each main node, the different robustness indices are indicated as follows: distance bootstrap support/maximum parsimony bootstrap support/maximum likelihood bootstrap support.

as the data matrix was saturated when sequences of *Mus* and *Rattus* (used as calibration points) were added. The analysis was therefore performed only using the *cytochrome b* region.

The ML distance between *Mus* and *Rattus*, which diverged 12 Mya, is 0.211. That between the subgenus *Apodemus* (*A. agrarius* and *A. peninsulae*) and the subgenus *Sylvaemus* (*A. mystacinus*), which diverged 7.9 Mya, is 0.167. These values give a rate of 0.0175–0.0211 (*Mus*/*Rattus* and *Apodemus*/*Sylvaemus*) ML distance per million years. When this rate is applied to the different dichotomies within *A. mystacinus*, the following molecular dates are obtained: 4.2–5.1 Myr for the separation between the Balkan and Oriental lineages, and 1.0–0.9 Myr for the separation between the animals from Crete/south-western Turkey and the other Middle East populations.

DISCUSSION

Taxonomy of *Apodemus mystacinus*

Johns & Avise (1998) stated that *cytochrome b* differentiation is highly congruent with traditional species boundaries. More recently, Bradley & Baker (2001) used a partition of genetic distances values (using the K2P parameters) in determining specific boundaries under the 'genetic species concept'. They also evidenced a strong correlation between genetic distance values and species boundaries, at least for different

Table 2. Degree of within and between clades sequence divergence (in per cent) with Kimura 2 parameters distance for the *cytochrome b* gene, D-loop and *IRBP* gene

Regions compared	Crete (B2)	South-western Turkey (B2)	Other Turkish regions Syria and Georgia (B1)	Greece (A)
Cytochrome b				
Crete (B2)	0.5			
South-western Turkey (B2)	0.9	0.7		
Other Turkish regions, Syria and Georgia (B1)	3.1	2.9	1.0	
Greece (A)	13.1	13.6	13.3	1.1
D-loop				
Crete (B2)	0.5			
South-western Turkey (B2)	0.85	1.0		
Other Turkish regions, Syria and Georgia (B1)	1.7	1.7	0.9	
Greece (A)	10.6	10.9	10.9	1.1
IRBP				
Crete (B2)	0.1			
South-western Turkey (B2)	1.0	0.3		
Other Turkish regions, Syria and Georgia (B1)	1.4	0.4	0.6	
Greece (A)	1.9	1.6	1.7	0.8

rodent and bat genera. Taking this concept into account and the values of genetic divergence observed between other closely related and well-defined *Apodemus* species such as *A. sylvaticus*, *A. flavicollis, A. hermonensis, A. alpicola* and *A. uralensis* (always 10–12% K2P distance, Michaux *et al*., 2002, 2003; 2004a, b), it can be assumed that the level of genetic divergence between clade 1 (Balkan population) and clade 2 (Turkish and near-east populations) (13.3% K2P distance) corresponds to different species. Similar results are obtained by considering the other genetic regions (Table 2).

Therefore, this strongly confirms Storch's (1977) and Mezhzherin's (1997) statements about the specific status of *A. epimelas* and *A. mystacinus*. These results are also consistent with the recent morphometric study of Vohralík *et al*. (2002) as well as with the allozyme research of Filippucci *et al*. (2002).

Moreover, they show for the first time, the existence of two well-separated genetic groups in Turkey and in the near-east. The first one corresponds to the populations from south-western Turkey and Crete; the second to all the other populations from Turkey, Syria and Georgia. They are separated by a quite important level of genetic divergence (3% K2P distance for *cytochrome b*), which suggests that they could be considered as different subspecies. Ondrias (1966) distinguishes a subspecies, *A. mystacinus rhodius*, in the islands of Crete, Karpathos and Rhodos, which is likely to be present also in south-western Turkey, considering these results. However, Vohralík *et al*. (2002), failed to show any morphometrical differentiation between these last populations as compared to the other oriental ones. Additional genetic but also morphological studies on further samples of Turkish *A. mystacinus* would be extremely useful to confirm this hypothesis.

Biogeography of *Apodemus mystacinus*

Appearance of A. mystacinus and A. epimelas

A. mystacinus appeared during the Middle Pliocene (Martín Suárez & Mein, 1998). During this period, the species was widespread throughout Europe, although with lower densities due to competition with many other rodents present at that time (Gliridae, Eomyidae, Sciuridae) (Michaux & Pasquier, 1974). It can therefore be assumed that one of the late Pliocene (4.2–5.1 Mya) climate change (maybe associated with the Messinian crisis), linked with low populations densities, led to the disappearance of many animals and the isolation of the two main groups, one in the Balkan region (where it is still confined at present; *A. epimelas*) and the other, *A. mystacinus s.s.* in the near and middle east (Turkey, Israel).

Although the Marmara Sea was frequently replaced by dry lands during the Quaternary ice ages, the European lineages do not seem to have invaded the oriental region and vice-versa (Vohralík *et al*., 2002). During the Quaternary, the two broad-toothed field mice lineages were probably established in the Balkan and Oriental regions a long time ago (Michaux & Pasquier, 1974). Therefore, each lineage could have prevented the colonization of invaders from the other. Indeed, once established, resident rodents often aggressively exclude newcomers (Granjon & Cheylan, 1989).

The presence of two genetic groups in Turkey and their relationship with the Cretan population

According to the mitochondrial DNA data (Fig. 2), two different genetic groups appear to live in Turkey: the first one corresponding to the majority of Turkish and Syrian populations (clade B1); the second one associating the animals from south-western Turkey (Antalya region) with those from Crete (clade B2). The divergence time analysis estimates a separation between them approximately 1–0.9 Mya. During this period, important climatic alterations arose throughout Europe and the near east (Fauquette, Guiot & Suc, 1998), so this structure could be explained by isolation of two groups of *A. mystacinus* in two different Turkish refugia (one in the south-western region, the second somewhere in another oriental region) during one of the Quaternary climatic oscillations. Indeed, as these climatic changes also altered the vegetation cover of the region (Borisova, 1993; Fauquette *et al*., 1998, 1999), the distribution of *A. mystacinus* in Turkey was probably deeply influenced during this period. Crete would have been colonized recently from animals living in south-western Turkey.

However, the nuclear *IRBP* gene does not show the same pattern at all, which suggests that the animals from Crete are well separated from the Turkish populations although they seem closely related. As *IRBP* genes evolve more slowly than the two mtDNA regions, it would better reflect an ancient and more complex evolutionary history of the broad-toothed field mouse in this region and suggest that a limited number of animals colonized Crete from Turkey a long time ago. This hypothesis is corroborated by the important number of DNA types (five different DNA sequences for five samples observed in the three studied genes) that are genetically weakly differentiated (Table 2), characterizing the Cretan population. Indeed, this kind of pattern generally corresponds to a recent genetic differentiation after an important ancient bottleneck or founder effect (Avise, 2000). The colonization of Crete from Turkish populations would have occurred via the Rhodes and Karpathos islands during one of the Quaternary ice ages, when the sea level decreased. When it rose again, *A. mystacinus* stayed isolated on the island for some time, leading to the emergence of a particular genetic lineage. It later invaded south-western Turkey from Crete, Rhodes and Karpathos during a new drop in sea level or via recent anthropogenic introductions and hybridized with south-western Turkish animals, giving them their particular mtDNA. This would explain the presence of two genetically differentiated mtDNA lineages in continental Turkey that are not evidenced by the nuclear gene.

However, this hypothesis is not corroborated by palaeogeographical and geodynamic data (Meulenkamp *et al*., 1988; Dermitzakis, 1990) attesting that Crete has not been connected with the mainland since the Early Pliocene, 5 Mya, even during the main dramatic Quaternary ice ages. Fossil records of *A. mystacinus* are also lacking in Crete (Kotsakis, 1990) despite well-documented palaeontological sites. Finally, as explained above, the colonization of Turkey from an insular lineage would have been difficult considering that an *A. mystacinus* population was already present in Turkey during the Quaternary period (Storch, 1977) and that they could have prevented the colonization of new invaders. This hypothesis is corroborated by the study of Darlington (1959), which suggested that the majority of islands were invaded by mainland taxa and not the reverse. This was the case for the spiny mouse, *Acomys minous*, which colonized Crete recently from oriental regions, via anthropogenic introductions associated with the important maritime traffic between Turkey and many eastern Mediterranean islands since the Bronze Age (Pulak, 1995; Barome *et al*., 2001). As far as another closely related species, the wood mouse *Apodemus sylvaticus*, is concerned, it seems that the colonists of Crete find their origin in Greece (J. Michaux, unpubl. data) and were also introduced recently by man.

Therefore, although our genetic data strongly suggest an ancient colonization of Crete by the broad-toothed field mouse followed by a secondary invasion of this insular population in continental Turkey, the question still remains open about the origin of the south-western Turkish genetic lineage. The study of a larger sample from this region, as well as from the islands of Rhodes and Karpathos, should help to conclude definitively about this question.

CONCLUSIONS

The phylogenetic relationships between the different lineages existing within *A. mystacinus* inferred from *cytochrome b*, D-loop and *IRBP* sequences show evidence for an important level of genetic divergence between the animals from the Balkans and those from Turkey and the near east. This is in favour of a distinctive specific status for these populations, namely *A. epimelas* and *A. mystacinus*, respectively. Moreover, the broad-toothed field mice from south-western Turkey appear to be closely related to the animals from Crete but clearly distinct from the populations of the other oriental regions. This supports a distinct subspecific level (*A. m. rhodius*) for the insular as well as for the south-western Turkish broad-toothed field mice.

From a biogeographical point of view, it can be assumed that one of the late Pliocene or early Pleistocene cooling periods led to the isolation of two main

groups of *A. mystacinus*: one in the Balkan region and the other one in Turkey and the near east (Syria, Israel). Moreover, it is suggested that the isolation of one population of *A. mystacinus* in Crete during one of the Quaternary ice ages would have led to the emergence of two well-differentiated genetic groups in the eastern Mediterranean region. One of these was in Crete and south-western Turkey, after a recent secondary invasion of the insular population in Turkey; the other in northern and eastern Turkey as well as in the near east. If this hypothesis is confirmed, the broad-toothed field mouse would be a rare example of current mammalian species that colonized the Mediterranean islands naturally without human intervention (Vigne, 1999).

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