On the mtDNA restriction patterns variation of the Iberian wood mouse (*Apodemus sylvaticus*). Comparison with other west Mediterranean populations

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In previous studies, the presence of three main lineages of wood mice mtDNA was described in western Europe: a first one distributed from the Pyrenees to Scandinavia, a Thyrrenian one occurring in peninsular Italy, Elba, Corsica and Sardinia and a third one restricted to Sicily and Marettimo. Do the Iberian wood mice belong to one of these lineages? In order to answer this question, animals were trapped all over the Iberian peninsula (11 sites) as well as in three of the Balearic islands. Comparisons with specimens from the above mentioned lineages were made. From 158 animals trapped in 30 sites, 78 different mtDNA restriction patterns were obtained and compared using the NEI and LI index ((Nei M and Li WH, (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76: 5269–5273) of nucleotide divergence. A neighbour-joining tree, with a specimen of *Apodemus flavicollis* as outgroup, was then computed from the similarity matrix. *Apodemus flavicollis* is well separated from all the *A. sylvaticus* and all the Iberian restriction patterns are clustered with those of continental France, showing a great similarity level between the Iberian and north-west European animals. This group is well separated from the Sicilian and from the Tyrrhenian ones. These results suggest that the Pyrenees are not a biogeographic barrier for the wood mouse and that the postglacial recolonisation of western Europe by that species has its origin in populations which, during the latest Ice age, were living in refuges situated in southern France or in the Iberian peninsula. Because the Balearic restriction patterns are clustered in a lineage joining Iberian patterns at a low divergence level, we suggest that their origin is continental Spain or southern France.

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In a previous study (MICHAUX et al. 1996), two lineages of wood mice (*Apodemus sylvaticus*) mtDNA were described in western Europe. The first one (northwestern) extends from the Pyrenean mountains to Scandinavia and the second one is distributed all over the Italian peninsula and in the Tyrrhenian islands (Elba, Corsica, Sardinia). A third group is recognised in Sicily and in Marettimo (MICHAUX et al. 1998).

Till now, no information about the genetic variability of the Iberian wood mouse mtDNA is available.

As the wood mouse is mainly a forest dweller whose altitudinal and latitudinal distribution boundaries are close to the tree limit, it is conceivable that during the latest Ice age, its distribution was restricted to the remaining forest areas of southern Europe e.g. in southern France and in some parts of the Italian and Iberian peninsulas.

Consequently, north-west Europe must have been reached by wood mice originating from these forest zones. Certainly, the alpine chain which constitutes the north-occidental boundary between the two main mtDNA lineages, has prevented the Italian animals to move northwards after glaciation. We wonder if the Iberian wood mice are distinct from the northwestern ones and if the Pyrenees played the same role as the Alps at the end of the last glaciation.

Two distinct subspecies are currently reported in the Iberian peninsula: *Apodemus sylvaticus dichrurus* (Rafinesque, 1814) considered as the Mediterranean subspecies and *A. s. callipides* (Cabrera, 1907) supposed to inhabit the Cantabric and the Pyrenean chains and even to extend towards the Massif Central (MILLER 1912; SAINT GIRONS 1966, 1973).

In their morphological study of Portuguese specimens, RAMALHINHO and MADUREIRA (1982) did not find any crucial difference between the north Portuguese animals thought to belong to *callipides* and the south Portuguese ones ascribed to *dichrurus*. Nevertheless, animals are slightly bigger in the south than in the north. This fact is highlighted by SANS-COMA et al. (1987) in Spain too. Despite that these morphological differences are slight, the existence of two distinct forms of the wood mouse in the Iberian peninsula cannot be excluded.

As far as the Balearic islands are concerned, it is well known that the endemic mammal fauna of the archipelago has been completely replaced by man-introduced species. Most of these introductions took place during the Holocene. From archaeozoological data, it is established that the presence of the wood mouse dates back to at least 6000 years B.P. in Mallorca and to 3300 y B.P. on Menorca (REUMER and SANDERS 1984; VIGNE and ALCOVER 1985), when human exchanges were already quite common between the archipelago and either the European or African coasts (GUILAINE 1976; CAMPS 1990). Consequently, different hypotheses about the geographic origin of the species in these islands can be inferred, especially if we consider that some small mammal insular parasites (fleas) undoubtedly have an African origin whereas others have clearly European or Eurasiatic affinities (BEAUCOURNU and ALCOVER 1984).

Owing to its overall bigger size and heavier weight, compared to continental references (ALCOVER and GOSALBEZ 1988), the wood mouse of Ibiza has received a distinct subspecific status: *A. s. eivissensis* ALCOVER 1977. Still heavier and bigger than those of Ibiza, the wood mice of Formentera were also described as a separate subspecies: *A. s. frumentariae* SANS-COMA and KAHMANN 1977. These taxonomic distinctions, however, give no information about the actual origin of the animals.

In this paper, we intend to study the mtDNA variability of Iberian wood mice, including animals from the Balearic islands and to compare their mtDNA restriction patterns with others from different continental areas. In this way, we hope to get more information about the influence of the Pyrenees on the postglacial recolonisation of Europe by the wood mouse, to know to what extent the Iberian populations of this species are differentiated and to understand the origin of its settlement in the Balearic archipelago.

MATERIAL AND METHODS

The wood mice and the yellow necked mouse were trapped in 'Manufrance' or in 'Sherman' traps previously baited. The sampling localities (two lines of 40 traps at 5 m. interval and distant of about 200 m. from each other) are spread all over Spain and Portugal as well as in Ibiza, Mallorca and Menorca. Unsuccessful attempts were also made on Formentera. A special effort of capture was made in the vicinity of La Coruña (*locus typicus* of the subspecies *A. s. callipides*) and also on the French slopes of the Pyrenees as well as in the Massif Central. Additional
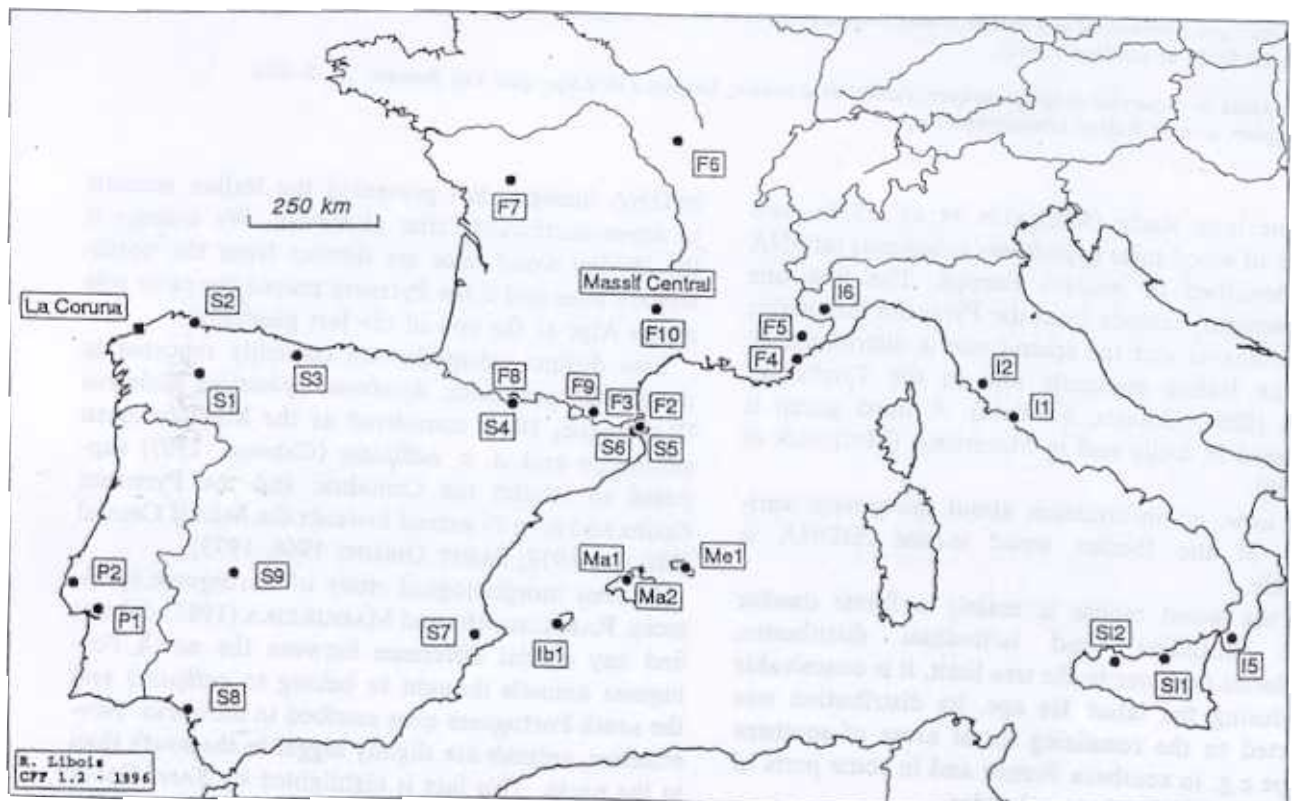

Fig. 1. Geographic distribution of the wood mice trapping localities. The codes refer to Table

Table 1. *Geographic coordinates (in centesimal degrees) and codes of the trapping localities*

Locality	Latitude	Longitude	No. animals	Locality code	Date M/Y
France					
Banyuls/Mer*	42.495	3.129	6	F2	February 93
Argelès (Massane)*	42.432	3.100	8	F3	May 93
Estérel (Mont Vinaigre)*	43.505	6.809	4	F4	May 94
La Penne*	43.894	6.941	2	F5	May 94
Saint Brisson*	47.266	4.094	11	F6	April 94
Ménigoute*	46.500	−0.046	2	F7	October 93
Fabian	42.765	0.203	4	F8	June 95
Bouillouses	42.579	2.029	2	F9	October 95
Ispagnac	44.372	3.533	2	F10	February 96
Italy					
Tarquinia*	42.260	11.745	26	I1	December 92
Grosseto*	42.880	11.090	2	I2	December 92
Gambarie*	38.135	15.867	4	I5	January 94
Cuneo*	44.345	7.500		I6	May 94
Sicily					
Ficuzza	37.919	14.400	3	Si1	January 94
Grateri	37.951	13.356	3	Si2	January 94
Portugal					
Setubal	38.575	−8.650		P1	March 96
Vale de Guarda	38.959	−9.265		P2	March 96
Spain					
As Nogais	42.784	−7.100	2	S1	May 95
Viveiro	43.635	−7.350	2	S2	May 95
Posada de Valdeon	43.257	−4.852	12	S3	May 95
Torla	42.676	0.182	2	S4	May 95
Figueras	42.270	2.981	7	S5	April 94
Llança	42.351	3.127	2	S6	April 94
Alcoy	38.703	−0.458	20	S7	May 95
Coto Doñana	37.081	−6.491		S8	January 96
Trujillo	39.473	−5.828		S9	March 96
Baleares					
Lluc	39.788	2.870	3	Ma1	October 97
Alamédra	39.729	2.818	6	Ma2	October 97
Alaior	39.958	4.154	4	Me1	October 97
Ibiza (3 sites)	38.941	1.290	10	Ib1	May 96

* The localities with an * are the same as in MICHAUX et al. (1996) and are numbered in accordance.

animals from France and Italy were included in the analyses to allow useful comparisons. Fig. 1 shows the geographic distribution of the sampling points and Table 1 gives information about the sample size and the precise location of each trapping site.

Trapped animals were maintained in the laboratory and killed to allow the isolation of mitochondrias from fresh tissue (heart, spleen, liver and kidneys) by differentiated centrifugations, according to LANSMAN et al. (1981). mtDNA was then isolated and purified by alkaline lysis and ether–phenol extraction (PALVA and PALVA 1985) and finally digested with two restriction enzymes: *Hae* III (GGCC) and *Rsa* I (GTAC) (Boehringer–Mannheim or BRL). One to 3 µl mtDNA solution containing 20–40 ng DNA were digested for 1–2 h in a 10 µl reaction volume containing one enzyme unit. The mtDNA fragments were

isolated by electrophoresis on 4% PAA gels according to TEGELSTRÖM (1986) and polymorphism visualised by the silver staining protocol of GUILLEMETTE and LEWIS (1983). All distinctive mtDNA restriction fragment patterns were assigned a number when produced by *Rsa* I or an alphabetical code when produced by *Hae* III. In this way, each animal was assigned an alphanumeric code. All specimens sharing the same composite restriction pattern were considered as belonging to the same mtDNA matrilineal clone.

The level of similarity between two individuals was computed using the NEI and LI (1979) index. A neighbour-joining tree was constructed from the similarity matrix using one individual of yellow-necked mouse (*A. flavicollis*) as outgroup. The robustness of the inferences was assessed through bootstrap analy-

sis (1000 repeats) (Treecon programme, version .2, VAN DE PEER and DE WACHTER 1996).

RESULTS

A total of 159 animals (158 *A. sylvaticus* and 1 *A. flavicollis*) representing 31 populations have been analysed using two restriction endonucleases. For each animal, we obtained a total of 42–46 fragments, a number which is appropriate for a reliable estimation of divergence levels among samples (FERRIS et al. 1983a,b). The tables of the different fragment sizes obtained after digestion by *Hae* III and *Rsa* I are available upon request from J.R.M.

We obtained 79 different haplotypes from the animals examined. Some of them are illustrated in Fig. 2 (*Hae* III) and Fig. 3 (*Rsa* I). No difference was observed in the total length of the mtDNA as recalculated after interpretation of each restriction pattern, a potential problem raised by TEGELSTRÖM and JAAROLA (1989). In Table 2, a haplotype diversity index (Shannon H' with log in base 2: SHANNON 1948) has been calculated. The obtained values are in strong correlation with sample size: $H' = 1.920 + 0.0504 \times$ nb of animals ($r = 0.999$). This is not the case with the regularity index (H'/H max) which is always very high, indicating that, for any sample size, the animals are quite uniformly distributed between the different haplotypes. A neighbour-joining tree (Fig. 4) shows that the yellow-necked mouse is very divergent of all the woodmice samples (mean level of genetic divergence: 6.27%). The node separating these

two species is very robust (BP of 100%). Moreover, inside the woodmice group, it can be seen that all the Iberian clones including the Balearic ones are clustered in a single subgroup including the French samples whereas Italian animals are separated in a second subgroup and Sicilian individuals in a third one. As expected from our previous study (MICHAUX et al. 1996), the mean level of the nucleotide sequence divergence between these three groups is relatively high (between 2.6 and 3.7%). This separation is very well supported with respective bootstrap values of 90 and 97%. The divergence within the Iberian/French group is low (1.1%), approximately the same as calculated previously for the 'north-western' group (0.8%) (MICHAUX et al. 1996).

Interestingly, quite different restriction patterns are found for animals caught at one locality, the divergence level between them being sometimes larger than 1% (e.g. patterns N26, L25, L27, O25, R24 in sampling point F2 or BB63, AZ58, AZ63, AZ70 and BA58 in point S3). Animals caught very far from each other sometimes share the same restriction pattern [e.g. pattern S29 in central France (point F6) and in Catalunya (point S5), or AQ 62 present in the central French Pyrenees (F8) and near Valencia (S7)].

Considering the islands, it can be seen that all the island clones are clumped together in a same subgroup and that the dominant pattern for *Rsa* I is the same (79) on Mallorca and Menorca. In fact, the island patterns are very similar to each other, leading to a particularly low level of genetic divergence between the insular animals (mean p = 0.3%).
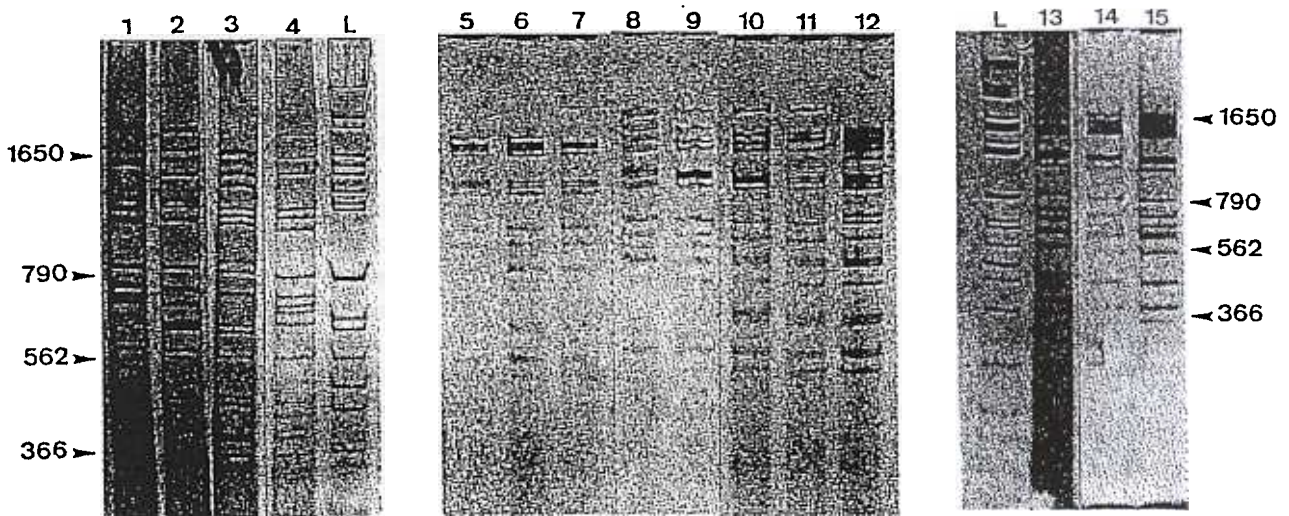

Fig. 2. Restriction patterns after *Hae* III endonuclease digestion of wood mouse mtDNA from 1: I5 (pattern A); 2: Ib1 (pattern V); 3: F7 (pattern Ag); 4: I1 (pattern A); 5: S7 (pattern Aq); 6: F8 (pattern Aq); 7: S3 (pattern Az); 8: Ib1 (pattern V); 9: Me1 (pattern Bj); 10: F4 (pattern An); 11: F5 (pattern Al); 12: F7 (pattern Ag); 13: Me1 (pattern Bj); 14 and 15: Ma2 (pattern Bl). The lanes marked L contains Lambda phage DNA digested with Restriction endonuclease *Bgl* I to produce fragment size markers (size in BP indicated on the right and on the left).
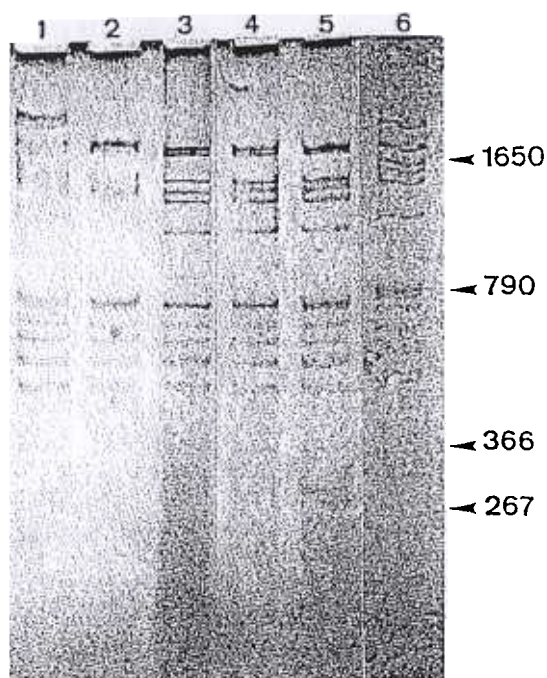
Fig. 3. Restriction patterns after *Rsa* I endonuclease digestion of wood mouse mtDNA from 1: Ma1 (pattern 80); 2: Me1 (pattern 79); 3: Ib1 (pattern 53); 4: S3 (pattern 63); 5: S5 (pattern 29) and 6: I1 (pattern 1). The size scale in BP is indicated on the right.

DISCUSSION AND CONCLUSIONS

The mean level of sequence divergence of nucleotides between Iberian and French animals is quite low and therefore Iberian animals can be considered as a part of the 'north-western' group whose distribution area extends to Scandinavia (TEGELSTRÖM and JAAROLA 1989; VAN ROMPAEY 1989; MICHAUX et al. 1996). These data strongly suggest that the post-glacial re-colonisation of north-west Europe is the consequence of the spreading of a clade which found refuge in the Iberian peninsula or in the very southern France during the latest Ice age. This colonisation route is identical for the wood mouse, the brown bear (*Ursus arctos*) and some white oaks (*Quercus spp.*) haplotypes (TABERLET et al., 1998) but differs for other species like the grasshopper *Chorthippus parallelus*

(COOPER et al., 1995) for which the Pyrenees are a suture zone between two groups of populations.

So, the Pyrenees do not constitute a serious obstacle for the wood mice north–south dispersal movements. The north–south orientation of the main valleys and the low altitude of many passes between the southern and the northern parts of the mountain chain leads to a continuity in the forest cover and perhaps favours the wood mouse settlement. Moreover, either near the Atlantic coast or near the Mediterranean border, this forest cover is also uninterrupted between France and Spain due to sufficient rainfalls, to suitable edaphic conditions and to the low altitude of the mountains. The situation prevailing in the Alps is contrasting, at least in the western part, because the summit line is higher, the altitude of only a few passes being just under or near the tree line. Near the Mediterranean sea, the piedmont is very dry and the soil is stony, leading to ecological conditions unsuitable for the wood mouse. In the past, the ice sheet was more developed in the Alps than in the Pyrenees, probably preventing the northward expansion of the mice populations isolated in the Italian refugium. This Alpine discontinuity has been evidenced in many other species including mammals (*Sorex araneus*, *Arvicola terrestris*: TABERLET et al., 1994; TABERLET et al. 1998), amphibians (*Triturus sp.*: WALLIS and ARNTZEN 1989), fish (*Salmo trutta*: BERNATCHEZ et al. 1992) or insects (*Chorthippus parallelus*: COOPER et al. 1995; *Apis mellifica*: GARNERY et al. 1992).

As far as the divergence level between the Iberian samples is very low, it can be assumed that there is no evidence for two distinct wood mice groups within the Iberian Peninsula. This conclusion is in complete accordance with the results of the morphological study of RAMALHINHO and MADUREIRA (1982). Moreover, a multivariate (PCA, multiple discriminant analysis) craniometric study (MICHAUX 1996) shows that the Iberian wood mice are hardly distinguished from those trapped in France, Belgium and Germany.

In conclusion, we suggest that the existence of *Apodemus s. dichrurus* in Spain and in southern

Table 2. *Woodmouse haplotype diversity in the different regions*

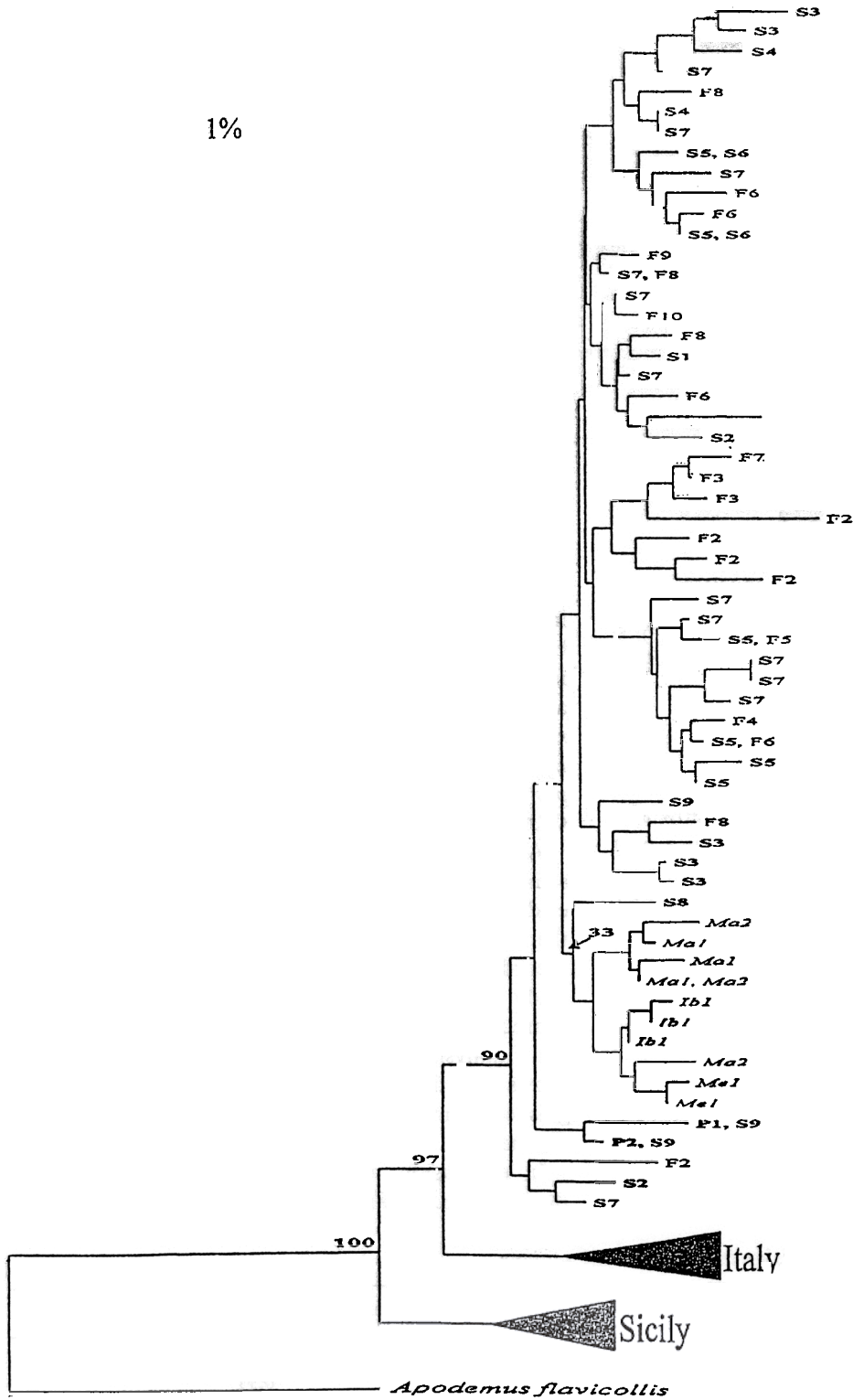|	No. haplotypes	No. animals	Diversity index	Equitability index
Sicily	5	6	2.252	0.969
Italy	15	33	3.555	0.91
France	21	41	4.047	0.921
Balearic islands	10	23	3.029	0.912
Iberian Peninsula	30	55	4.686	0.955
Id. + France	48	96	5.304	0.95

Fig. 4. Neighbour joining tree of the 79 *Apodemus* haplotypes. The Sicilian and Italian subgroups are presented as single branches. Number over branches indicate bootstrap support (1000 replicates).

France has to be rejected since this taxon is described from Sicily and because the Sicilian mtDNA restriction patterns form a particular lineage, which is separated from both the Italian and the north-western ones at a high divergence level (MICHAUX et al. 1998 and this study). Furthermore, the animals

trapped near the *locus typicus* of *A. s. callipides* reveal restriction patterns which are very similar to those of other Spanish or French animals. Therefore, and following CORBET (1978), we propose to consider *A. s. callipides* as an invalid subspecies and to put it in synonymy with the nominal one.

The restriction patterns found in the Balearic animals are clearly of the 'north-western' type, indicating that the origin of introduced animals is the Spanish or French mainland, provided similar mtDNA restriction patterns are not present in northern Africa. The genetic similarity observed within and between the populations of neighbouring islands as well as their grouping in a separate branch of the cladogram suggests a small founder population.

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