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Phylogeography of the false smooth snakes, *Macroprotodon* (Serpentes, Colubridae): mitochondrial DNA sequences show European populations arrived recently from Northwest Africa

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Abstract

Mitochondrial DNA (1075 bp: cytochrome *b*, 300 bp; 12S rRNA, 393 bp; and 16S rRNA, 382 bp) corroborates the monophyly of the genus *Macroprotodon* and of the species *M. mauritanicus*, *M. abubakeri*, and *M. brevis*. The subspecies *M. brevis ibericus* is also monophyletic. The mtDNA tree presented here indicates that *M. cucullatus* consists of at least two separate units and may possibly represent a primitive morphology rather than a species in its own right. However, this hypothesis is tentative since it is only reflects the history of a single evolutionary unit (mtDNA). A definitive understanding of the evolution of *M. cucullatus* will not be possible until informative nuclear markers are added to the mitochondrial data. *Macroprotodon* appears to have originated in the Maghreb region of NW Africa and speciated there around 4–5.5 million years ago around the end of the Miocene period, after which its three main lineages may each have expanded north into more mesic conditions. The group also spread eastwards into coastal areas of Libya quite recently and on to Egypt and Israel. Later still, *M. b. ibericus* from extreme north Morocco reached the Iberian Peninsula, and *M. mauritanicus* from Tunisia or Algeria colonised the Balearic Islands of Menorca and Mallorca. Both these range extensions may result from very recent natural colonisations or even from accidental human introduction. Recency of origin of Iberian and Balearic populations is indicated by uniformity of their mtDNA even across large distances, and its great similarity to that of populations in source regions. Isolated populations assigned to *M. cucullatus* in the Hoggar mountains (southern Algeria) and Western Sahara are probably relicts from quite recent periods of climatic amelioration in the North African desert.

Keywords: Mitochondrial DNA; Cytochrome *b*; 12S rRNA; 16S rRNA; Evolution; Phylogeography; Dispersal; Colonisation

1. Introduction

The Mediterranean basin between southern Europe and North Africa has a complicated history, which largely results from the northward Tertiary movement of Africa towards western Eurasia. The eastern end of the Mediterranean closed in the mid-Miocene period 15–19 million years ago (Rögl, 1999), when the northeast part of the African plate collided with the Turkish plate, permitting a biotic interchange between nearby areas of Eurasia and Africa (Wolfart, 1987). A second, although brief contact arose from events at the other extremity of the Mediterranean, right at the end of the Miocene about 5.6 million years ago, when this sea became separated

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from the Atlantic Ocean and temporarily desiccated in a series of events called the Messinian salinity crisis. Again, there was biotic interchange, this time between Europe and northwest Africa, but it was followed by fragmentation of the extended ranges of the taxa involved when Mediterranean contact with the Atlantic was re-established 5.3 million years ago (Carranza and Arnold, 2004).

The events at the western end of the Mediterranean are likely to have left their imprint on the phylogenies of many of the taxa concerned, as they have in the newts of the genus *Pleurodeles* (Carranza and Arnold, 2004; Veith et al., 2004). In this paper, the present taxonomy of another of the 22 reptile and amphibian groups present on both sides of the Strait of Gibraltar, the False smooth snakes, *Macroprotodon*, is tested and its history and phylogeography examined using 1075 bp (base pairs) of mitochondrial DNA sequence (cytochrome *b*, 12S rRNA, and 16S rRNA).

Macroprotodon are colubrines that are found in mainly Mediterranean areas of North Africa, the Iberian Peninsula (Iberia) and on some Western Mediterranean islands including Mallorca, Menorca, Galita, Lampedusa, Djerba, Zembra, Zembretta, and Habibas (Doumergue, 1901; Lanza and Bruzzone, 1959, 1960; Marinkelle, 1962; Busack and McCoy, 1990; Joger, 1999; Wade, 2001). Isolated populations are found in Israel, the Hoggar (South Algeria), southern Morocco, and coastal Western Sahara (Fig. 1). *Macroprotodon* feed mainly on lizards, and are small, mainly nocturnal and rather secretive, features that have resulted in their being relatively poorly known (Pleguezuelos et al., 1994). The taxonomy of the genus has been revised three times in recent years (Wade, 1988, 2001; Busack and McCoy, 1990). The most recent revision (Wade, 2001) used a range of morphological features including scaling, coloration and number of premaxillary teeth. Four species are recognised (Fig. 1): *Macroprotodon cucullatus* (Geoffroy de St Hilaire, 1827) occurs in relatively arid areas of North Africa, while *M. mauritanicus* Guichenot, 1850, *M. abubakeri* Wade, 2001, and *M. brevis* (Günther, 1862) are found mainly further north in rather more mesic regions. *M. cucullatus* is divided into eastern *M. c. cucullatus* and western *M. cucullatus textilis* (Duméril and Bibron, 1854), while *M. brevis* is separated into a Moroccan *M. b. brevis* and *M. b. ibericus* (Busack and McCoy, 1990); which occurs in extreme northern Morocco and Iberia. A North African origin for *Macroprotodon* has been postulated, with movement into Iberia before the opening of the Straits of Gibraltar at the end of the Miocene period (Pleguezuelos et al., 1994).

2. Material and methods

2.1. Samples and DNA extraction

Thirty-four individuals of *Macroprotodon* including representatives of all recognised taxa were included in the analyses, together with five other species of colubrines (*Lytorhynchus diadema*, *Coluber viridiflavus*, *Coluber algirus*, *Coluber hippocrepis*, and *Coronella girondica*), and three psammophines that were used as outgroups (*Psammophis schokari*, *Malpolon moilensis*, and *M. monspessulanus*). Details of localities, sources, and GenBank numbers for all these materials are given in Table 1 and localities shown in Fig. 1.

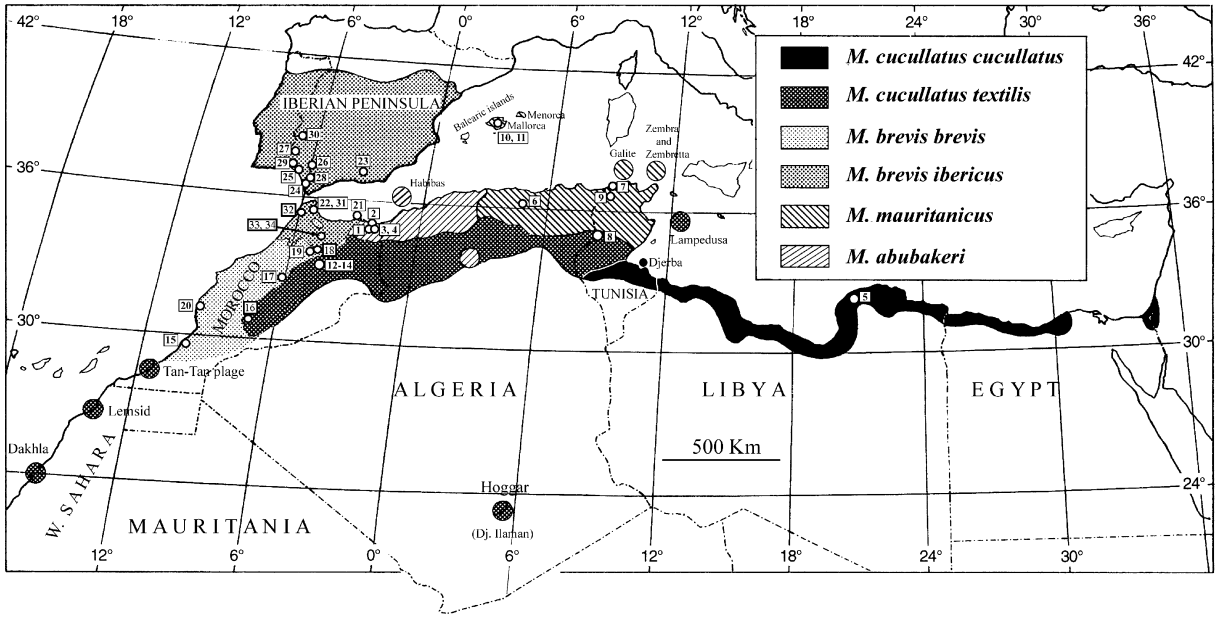

Fig. 1. Present taxonomy of *Macroprotodon* (Wade, 2001) showing the geographical distribution of species and subspecies and localities of all the samples used in the molecular analysis. Numbers refer to Table 1.

Table 1
Details of material and sequences used in the present study

Taxa	Specimen code (Fig. 1)	Locality	GenBank Accession Nos. (cyt*b*/12S/16S)	Specimen code
Macroprotodon abubakeri	1	Taforalt (Morocco)	AY643380/AY643297/AY643338	E2208.2
Macroprotodon abubakeri	2	E. Molouya estuary (Morocco)	AY643381/AY643298/AY643339	E608.1
Macroprotodon abubakeri	3	Beni Snassen (Morocco)	AY643382/AY643299/AY643340	E2901.3
Macroprotodon abubakeri	4	Beni Snassen (Morocco)	AY643383/AY643300/AY643341	E2901.4
Macroprotodon cucullatus cucullatus#	5	El Agheila (Lybia)	AY643390/----------/AY643348	E2208.5
Macroprotodon mauritanicus	6	Médjana (Algeria)	AY643389/AY643306/AY643347	E2208.4
Macroprotodon mauritanicus	7	Tabarka (Tunisia)	AY643386/AY643303/AY643344	E608.3
Macroprotodon cucullatus textilis#	8	Bou Chebka (Tunisia)	AY643387/AY643304/AY643345	E608.5
Macroprotodon mauritanicus	9	Ain Draham (Tunisia)	AY643388/AY643305/AY643346	E608.2
Macroprotodon mauritanicus	10	Mallorca, Baleanc I. (Spain)	AY643384/AY643301/AY643342	E2208.6
Macroprotodon mauritanicus	11	Mallorca, Baleanc I. (Spain)	AY643385/AY643302/AY643343	E1110.12
Macroprotodon cucullatus textilis#	12	Amersid (Morocco)	AY643371/AY643288/AY643329	E2208.3
Macroprotodon cucullatus textilis	13	Amersid (Morocco)	AY643372/AY643289/AY643330	E1110.13
Macroprotodon cucullatus textilis	14	Amersid (Morocco)	AY643373/AY643290/AY643331	E1110.14
Macroprotodon brevis brevis	15	Sidi Ifni (Morocco)	AY643378/AY643295/AY643336	E2208.8
*Macroprotodon cucullatus textilis**	16	Close to Tizi-n-Tichka (Morocco)	AY643379/AY643296/AY643337	5113.7
Macroprotodon brevis brevis	17	Naour (Morocco)	AY643374/AY643291/AY643332	E2208.7
Macroprotodon brevis brevis	18	Ifrane (Morocco)	AY643375/AY643292/AY643333	E5113.11
Macroprotodon brevis brevis	19	Azrou (Morocco)	AY643376/AY643293/AY643334	E2208.1
Macroprotodon brevis brevis	20	16Km. S. of Essaouira (Morocco)	AY643377/AY643294/AY643335	E608.4
*Macroprotodon brevis ibericus**	21	Cap des Trois Fourches (Morocco)	AY643370/AY643287/AY643328	E2901.2
Macroprotodon brevisibericus	22	Tétouan (Morocco)	AY643366/AY643283/AY643324	E2901.1
Macroprotodon brevis ibericus	23	Albunuelas (Spain)	AY64335/AY643274/AY643315	E512.42
Macroprotodon brevis ibericus	24	Barbate (Spain)	AY643358/AY643275/AY643316	1110.15
Macroprotodon brevis ibericus	25	Mazagón (Spain)	AY643359/AY643276/AY643317	E512.41
Macroprotodon brevis ibericus	26	Gandul (Spain)	AY643360/AY643277/AY643318	E512.40
Macroprotodon brevis ibericus	27	Puebla de Guzman (Spain)	AY643361/AY643278/AY643319	E512.39
Macroprotodon brevis ibericus	28	Benaocaz (Spain)	AY643362/AY643279/AY643320	E512.38
Macroprotodon brevis ibericus	29	Huelva (Spain)	AY643363/AY643280/AY643321	E608.6
Macroprotodon brevis ibericus	30	Valle de Matamoros (Spain)	AY643364/AY643281/AY643322	E512.37
Macroprotodon brevis ibericus	31	Tétouan (Morocco)	AY643365/AY643282/AY643323	E512.36
Macroprotodon brevis ibericus	32	Ashila (Morocco)	AY643367/AY643284/AY643325	E5113.8
Macroprotodon brevis ibericus	33	Fez (Morocco)	AY643368/AY643285/AY643326	E5113.9
Macroprotodon brevis ibericus	34	Fez (Morocco)	AY643369/AY643286/AY643327	E5113.10
Psammophis schokari		East of Debdou (Morocco)	AY643398/AY643314/AY643356	E1110.31
Malpolon moilensis		Berhoun (Algeria)	AY643397/AY643313/AY643355	E1110.16
Malpolon monspessulanus		Arba-des-Beni-Hassan (Morocco)	AY643396/AY643312/AY643354	E2509.15
Lytorhynchus diadema		Egypt	AY643393/AY643309/AY643351	E2509.11
Coluber hippocrepis		Zinat (Morocco)	AY643392/AY643308/AY643350	E2509.2
Coluber algirus		Bou Chebka (Tunisia)	AY643391/AY643307/AY643349	E1110.1
Coluber viridiflavus		Corsica (Italy)	AY643394/AY643310/AY643352	E1110.9
Coronella girondica		Cudia Adru, Yebala (Morocco)	AY643395/AY643311/AY643353	E512.20

Specimen codes identify each individual sequenced (Fig. 2) and its locality (Fig. 1). Specimens marked with an * are deposited in the Département de Biologie, Faculté des Sciences, Université Abdelmalek Essaâdi, Tétouan, Morocco. These marked with a # are deposited in the Natural History Museum, London.

Genomic DNA was extracted from approximately 1–3 mm^3 of tissue from each individual following standard protocols described elsewhere (Carranza et al., 2000). Primers used in both amplification and sequencing were CBV14846F1 (Lenk et al., 2001) and cytochrome *b2* (Kocher et al., 1989) for the cyt*b* gene, 12Sa and 12Sb for the 12S rRNA gene (Kocher et al., 1989), and L2510 and H3062 for the 16S rRNA gene (Lenk et al., 2001). The three gene fragments were amplified using the PCR procedures described by Carranza et al. (2000) and processed with an ABI 377 automated sequencer following the manufacturer's protocols. All three genes were sequenced for nearly all specimens, the only exception being the 12S rRNA of a *M. c. cucullatus* from Libya (Table 1, specimen 5), which could not be amplified despite the use of different PCR conditions and more specific PCR primers (data not shown).

2.2. Phylogenetic analyses

DNA sequences were aligned using ClustalX (Thompson et al., 1997) with default parameters. No gaps were included in the cytochrome *b* (cyt*b*) alignment and no stop codons were observed when the sequences were translated into amino acids using the vertebrate mitochondrial code, suggesting that all the cyt*b* sequences analysed were functional. Fifteen gaps had to be postulated to align all 12S rRNA sequences and no regions were excluded from the analysis. For the 16S rRNA, a region of 26–31 bp could not be unambiguously aligned. Outside this hypervariable region, only three gaps had to be postulated to align all 16S rRNA sequences. The incongruence length difference (ILD) test (Mickevich and Farris, 1981; Farris et al., 1994) was used to check for incongruence between the three gene fragments. In this test, 10,000 heuristic searches were made and invariable characters were removed beforehand (Cunningham, 1997).

Three methods of phylogenetic analysis were employed for all analyses of separate gene fragments and for two analyses in which they were combined. These were: maximum-likelihood (ML), Bayesian analysis, and maximum-parsimony (MP). Modeltest (Posada and Crandall, 1998) was used to select the most appropriate model of sequence evolution for the ML and Bayesian analyses, under the Akaike Information Criterion. This was the general time reversible model (GTR) taking into account the shape of the gamma distribution (G) and the number of invariable sites (I). Bayesian phylogenetic analyses were performed with MRBAYES v. 3.0b4 (Huelsenbeck and Ronquist, 2001) using the GTR+G+I model of sequence evolution (see above) with parameters estimated as part of the analysis and four incrementally heated Markov chains with the default heating values. All analyses started with randomly generated trees and ran for 1.5×10^6 generations, with sampling at intervals of 100 generations that produced 15,000 sampled trees. To ensure that the analyses were not trapped on local optima, the data set was run three times independently, each run beginning with a different starting tree. The log-likelihood values of the 15,000 trees in each analysis were plotted against the generation time. After verifying that stationarity had been reached, both in terms of likelihood scores and parameter estimation, the first 5000 trees were discarded in all three runs and a majority rule consensus tree was generated from the remaining 10,000 (post-burnin) trees. The frequency of any particular clade among the individual trees contributing to the consensus tree represents the posterior probability of that clade (Huelsenbeck and Ronquist, 2001); only values above 95% were regarded as indicating that clades were significantly supported (Wilcox et al., 2002).

Both ML and MP analyses were performed in PAUP* 4.0b10 (Swofford, 1998) and included heuristic searches involving tree bisection and reconnection (TBR) branch swapping with 10 and 100 random stepwise additions of taxa, respectively. Gaps were included as a fifth state. Independent MP analyses of separate gene fragments were carried out giving the same weight to ts and tv and also including the following corrections for the observed ts/tv ratios: cyt*b* (ts = 1; tv = 6); 12S rRNA (ts = 1; tv = 4) and 16S rRNA (ts = 1; tv = 2). In MP analyses involving all gene fragments, transversions (tv) were given the same weight as transitions (ts), and four times that weight in order to correct for the observed ts/tv ratio (ts/tv = 4.3). Robustness of the MP results was assessed by bootstrap analysis (Felsenstein, 1985) involving 1000 pseudo-replications.

To test some relationships, topological constraints were generated using MacClade v. 4.0 (Maddison and Maddison, 1992), and compared to optimal topologies, produced by phylogenetic analysis, using the Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) implemented in PAUP* 4.0b10 (Swofford, 1998).

3. Results

Independent MP, ML, and Bayesian analyses of each of the three gene fragments produced trees that differed only slightly. They varied only in such features as the arrangement of some colubrines outside *Macroprotodon*, which of the three clades of *Macroprotodon* revealed in all trees was most basal, and minor differences in the position of individual samples. None of the variations had strong bootstrap and posterior probability support. As the data also passed an incongruence length difference (ILD) test ($p > 0.35$), all three genes were combined in two further phylogenetic analyses, in which the 16S rRNA hypervariable region (see above) was either included or excluded. The resultant trees were almost identical. Excluding the 21–31 bp hypervariable region of 16S rRNA, a total of 1075 bp of mtDNA sequence were analysed (cyt*b*, 300 bp; 12S rRNA, 393 bp; and 16S rRNA, 382 bp). Of these, 330 were variable and 241 parsimony–informative, the respective numbers for each gene fragment being: cyt*b*, 132 and 100; 12S rRNA, 129 and 94; and 16S rRNA, 69 and 47. A saturation analysis for each of the third-codon positions of the cyt*b* and the 12S and 16S rRNA genes was carried out by plotting the observed proportions of transitions (ts) and transversions (tv) against uncorrected genetic distances. Not even the highly variable cyt*b* third codon transitions exhibited any sign of saturation (data not shown) justifying the inclusion of all positions in the analyses.

The analysis of the combined data set excluding the 16S rRNA hypervariable region presented in Fig. 2, and all other analyses (see above), indicate that *Macroprotodon* is monophyletic with respect to the other snake taxa included. It consists of three main clades, each made

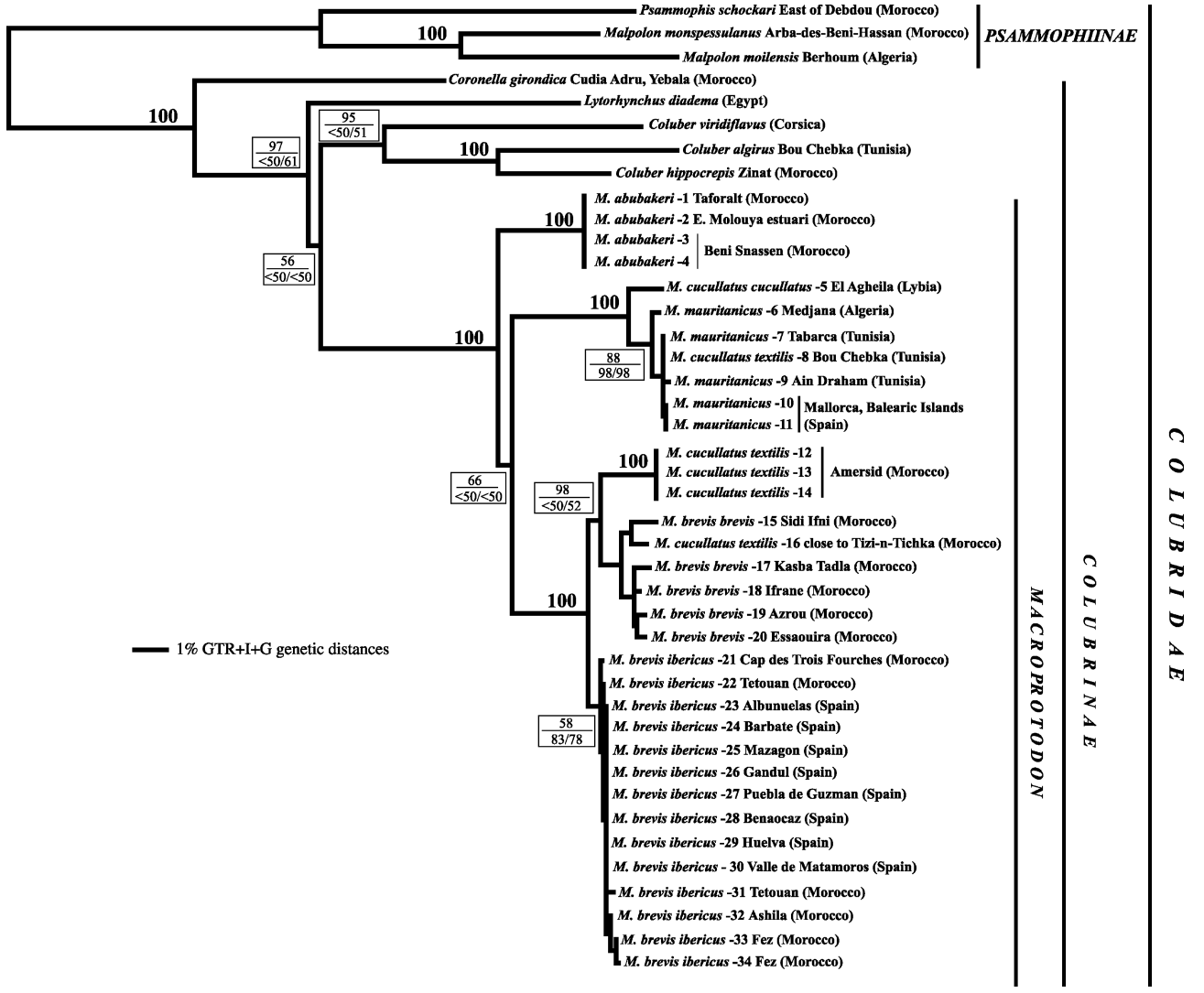

Fig. 2. ML Phylogenetic tree of *Macroprotodon* (−Log likelihood = 4832.97250) inferred from cyt*b* + 12S rRNA + 16S rRNA mitochondrial genes together (1075 bp). Bootstrap support and posterior probability values are shown at the corresponding nodes in boxes: upper figure is posterior probability from Bayesian analysis (mean of all three replicates); lower left, bootstrap support derived by MP (ts = tv) and lower right, bootstrap support derived by MP (ts = 1; tv = 4). When differences between the three support values or between all three Bayesian posterior probabilities was <5%, only the average value is shown.

up largely or wholly of individuals assigned on morphological grounds to a different species, namely *M. abubakeri*, *M. mauritanicus*, and *M. brevis*. In the ML, Bayesian and MP (ts = tv) analyses *M. abubakeri* is sister to all other *Macroprotodon*, but in the MP (ts = 1; tv = 4) analysis this basal position is occupied by a clade formed by *M. mauritanicus* plus two *M. cucullatus* (specimens 5 and 8; see Fig. 2). The fact, that these two alternative topologies each has only very low bootstrap and posterior probability support, may indicate the speciation events that produced the three main *Macroprotodon* clades occurred within a very short time span. Alternatively, this may be the effect of the length and evolutionary rate of the different molecules used in this study.

The six animals assigned to *M. cucullatus* included in the analyses do not form a monophyletic group. As noted, two are associated with *M. mauritanicus* (a *M. c. cucullatus* from Lybia and a *M. c. textilis* from Tunisia, respectively specimens 5 and 8 in Table 1) and the remainder form a clade with *M. brevis*. (four *M. c. textilis* from Morocco, specimens 12–14 and 16 in Table 1). The Moroccan *M. c. textilis* are specifically related to *M. b. brevis*, an association supported by all three phylogentic methods employed with a posterior probability of 98 in Bayesian analysis. When all six *M. cucullatus* and the five *M. c. textilis* were constrained to monophyly in an independent ML (GTR+I+G) analysis and the resultant trees compared with the one in Fig. 2, a SH test rejected the monophyly of both *M. cucullatus* as a whole and of *M. c. textilis* (Table 2).

All four specimens of *M. abubakeri* were collected in the same area of northeastern Morocco (see Fig. 1) and

Table 2
Statistical support for alternative hypotheses of phylogenetic relationships of *Macroprotodon*

Tree	−log likelihood	Δ − log likelihood	SH *p*
Unconstrained ML tree (Fig. 2)	4832.97250	(best)	
M. cucullatus constrained to monophyly	4994.38175	161.40925	0.0000*
M. cucullatus textilis constrained to monophyly	4989.72684	156.75434	0.0000*

SH, Shimodaira–Hasegawa test; * indicates $p < 0.001$ and suggests that the constrained and unconstrained trees are significantly different.

are identical in the 1075 bp of mitochondrial DNA analysed. The paraphyletic group comprising *M. b. brevis*, *M. b. ibericus*, and four Moroccan *M. c. textilis* is very well supported in all analyses. Despite covering a wide geographical area, all samples of *M. b. ibericus* from Spain are identical to each other in the mitochondrial gene fragments used here. They are also very similar to ones from Tétouan (specimens 22 and 31 in Table 1), Cap des Trois Fourches (specimen 21 in Table 1), Fez (specimens 33 and 34 in Table 1), and Ashila (specimen 32 in Table 1), which cover much of the distribution of *M. b. ibericus* in Morocco (see Fig. 1). The two samples of *M. mauritanicus* from Mallorca are also genetically very similar (maximum of two base pair differences in 1075 bp) to members of this species from Tunisia (specimens 7 and 9 in Table 1) and to a snake assigned to *M. c. textilis* from Bou Chebka, Tunisia (specimen 8 in Table 1).

Unfortunately, there is at present no means of internally calibrating a molecular clock for *Macroprotodon* itself, or for a larger clade that also includes its closer relatives. Consequently, only a rough estimation of divergence rates can be made using more distant relatives among the Squamata. Evolutionary rates calculated for exactly the same region of cyt*b* used in *Macroprotodon* range from 2.2% per million years in lacertid lizards, *Gallotia* (Maca-Meyer et al., 2003) to approximately 2.6% per million years in gekkonid lizards, *Tarentola* (Carranza et al., 2002) and 3.2% per million years in scincid lizards, *Chalcides* (S. Carranza and E.N. Arnold, unpublished data). The mean Kimura 2-parameter (K2P) sequence divergence of the cyt*b* gene fragment used in this study, between all three main *Macroprotodon* groups, is approximately 12% (Table 3). If rates in other squamates were applicable to *Macroprotodon*, the three main assemblages within the genus would have diverged about 4–5.5 million years ago, during the early Pliocene period or perhaps in the Messinian stage at the very end of the Miocene.

4. Discussion

4.1. Species boundaries

As noted, mitochondrial DNA strongly supports three monophyletic units within *Macroprotodon*: the newly described *M. abubakeri* and clades consisting largely of *M. mauritanicus* and *M. brevis*. In contrast, individuals assigned to *M. cucullatus* on the basis of morphology are associated with either *M. mauritanicus* or *M. brevis*, making these paraphyletic and indicating that the *M. cucullatus* specimens cannot be regarded as belonging to a single monophyletic species. The characteristic *M. cucullatus* morphology may have arisen two or more times or it may represent a primitive condition that has changed within both *M. mauritanicus* and *M. brevis*. This putative shift would involve relatively few alterations including a reduction in the average number of premaxillary teeth, changes in colouration and, in the case of *M. brevis*, an increase in the number of dorsal scales (see details in Wade, 1988, 2001; Busack and McCoy, 1990). If this interpretation is correct, it is possible that similar changes occurred within *M. abubakeri*, but no DNA samples are available from populations assigned to *M. cucullatus* that border the range of this species and which might be basal to it.

Another possible explanation is that all *M. c. textilis* populations sampled in this study have undergone intro-

Table 3
Kimura two-parameter genetic distances for the cyt*b* gene only among all main clades within *Macroprotodon* (see Figs. 1 and 2)

	1 *abubakeri* (%)	2 *mauritan.* (%)	3 *brevis* (%)	4 *ibericus* (%)	5 *cucullatus* (Lybia) (%)	6 *textilis* (Morocco) (%)	Internal variability (%)
1. *M. abubakeri*	—						0.0
2. *M. mauritanicus*	12.4	—					0.4
3. *M. b. brevis*	8.9	14.7	—				2.6
4. *M. b. ibericus*	10.8	15.3	4.7	—			0.3
5. *M. c. cucullatus* (Libya)	10.9	4.1	13.0	13.3	—		—
6. *M c. textilis* (Morocco)	12.4	14.4	5.9	5.3	13.6	—	3.3
7. *M c. textilis* (Tunisia)	12.2	0.2	14.7	15.5	4.1	14.6	—

gression and now have mitochondrial genomes originating in neighbouring species (*M. b. brevis* for specimens 12–14 and 16 from Morocco and *M. mauritanicus* for specimen 8 from Tunisia). The incorporation of genes of one species into another is a well-documented phenomenon and, if undetected, can prevent the retrieval of the true evolutionary relationships (Ruedi et al., 1997; Alves et al., 2003). This is especially likely when phylogenies are inferred using either mitochondrial or nuclear genes but not a combination of both, the situation in the current study. On present evidence, the possibility of introgression cannot be excluded as the sample of *M. c. textilis* used is quite restricted and comes from localities close to the distributions of *M. brevis* and *M. mauritanicus*. A proper understanding of *M. cucullatus* must await the availability of DNA samples from a much wider range of populations, including ones distant from *M. brevis* and *M. mauritanicus* such as the isolates in the Hoggar (Algeria) and Western Sahara and populations close to the type locality of *M. cucullatus* in Lower Egypt. Informative nuclear markers also need to be included in future studies of *Macroprotodon*.

4.2. Biogeography

The most parsimonious interpretation of the phylogeny presented in Fig. 2 is that the ancestor of the current species of *Macroprotodon* speciated in the Mahgreb region of North Africa (Tunisia, north Algeria and Morocco) 4–5.5 million years ago. From here there was relatively recent spread eastwards into Libya and ultimately Egypt and Israel and, still later, independent expansion into the Iberian Peninsula and the Balearic islands. An African origin is also suggested by molecular evidence that *Macroprotodon* may be closely related to African colubrines, including *Coluber dorri* of Senegal (Nagy et al., 2003).

Molecular data corroborate morphological evidence (Wade, 2001; Wade, unpublished data) that Iberian *Macroprotodon* are most closely related to ones in northwest Morocco, with which they are included in the subspecies *M. b. ibericus*. There is only one difference in 1075 bp between the eight Iberian animals studied here and Moroccan samples of *M. b. ibericus* from Tétouan and Ashila (samples 22 and 32 in Fig. 1; see also Fig. 2). Electrophoretic studies of enzymes also suggest a close relationship between Iberian and northern Moroccan snakes, the genetic distance between them, $D \pm SE$ (Nei, 1978), being only 0.03 ± 0.03 (Busack and McCoy, 1990). Moroccan *M. brevis* are also paraphyletic with respect to Iberian animals and this, together with lack of genetic variability in the latter corroborates evidence from the topology of the *Macroprotodon* phylogeny as a whole that the Iberian peninsula was colonised from Africa. The alternative possibility, that the ancestor of *M. b. ibericus* first colonised Iberia from Africa and then the subspecies reinvaded Morocco, is much less parsimonious. The dispersal of *Macroprotodon* across the Strait of Gibraltar is not an isolated example. Studies of other groups, such as the newt *Pleurodeles* and the lizards *Podarcis* and *Lacerta*, indicate that the presence of these taxa on both sides of the strait also best better explained by this mechanism (Carranza and Arnold, 2004; Harris et al., 2002; Paulo, 2001).

Similarity of mtDNA in northwest Moroccan and Iberian populations, and lack of variation in the latter in contrast to Morocco as a whole, suggests colonisation was recent. Invasion of the Iberian peninsula could not have been across land during the terrestrial contact with Africa 5.6–5.3 million years ago in the Messinian Salinity Crisis. Had *Macroprotodon* reached Iberia at this time, a substantial genetic divergence between Iberian and North Moroccan animals would be expected. If the evolutionary rate of 2.2–3.2% for the fragment of cyt*b* used here (p. 6) is accepted, this would be about 11–17% but is actually 0.01%.

Pliocene fossils from Spain have been assigned to *Macroprotodon*, but their identity is not certain. A few vertebrae from the mid-Pliocene of Layna in Soria province have been considered to be most similar to *Macroprotodon* (Szyndlar, 1988), but resemblance to *Coronella* was also noted (Jaén and Sanchiz, 1985). Fragmentary material from the upper-Pliocene of the Medes Islands off the coast of Girona, northeast Spain was assigned to *Macroprotodon* (Bailón, 1991), but was not mentioned in a later publication about the same site (Bailón, 1992). If *Macroprotodon* were really present in the Iberian peninsula during the Pliocene, it has now clearly been replaced by *M. b. ibericus* (see Fig. 2), that arrived from Morocco much more recently.

Although the *M. b. ibericus* from the Iberian peninsula investigated here are very uniform in their mitochondrial DNA, snakes in this area do exhibit some morphological variability. For instance, there is often a strong tendency for reduction of longitudinal dorsal scale rows from 21 to 19, and of upper labial scales from 8 to 7 (Wade, 2001; Wade, personal observations). Electrophoretic studies of enzymes (Busack and McCoy, 1990) also reveal some variation within Iberia (14.6% of 41 loci polymorphic; 0.016 average heterozygosity). But variation is lower than in Moroccan *M. b. ibericus* (26.8% of 41 loci polymorphic; 0.016 average heterozygosity), even though the number of individual snakes used was higher (12 from Spain compared with only five from Morocco). These results correlate with the greater variation of mtDNA within Moroccan *M. b. ibericus*. The variability revealed by electrophoresis suggests that the Iberian populations may have originated from several individuals and perhaps resulted from more than one colonisation rather than from a single propagule.

Macroprotodon may have reached the Iberian Peninsula by recent natural transmarine colonisation, which is

a widespread phenomenon in reptiles (Carranza and Arnold, 2003; Carranza et al., 2000, 2001; Arnold and Carranza, work in progress). This however raises the question of why colonisation took so long to occur, considering that *Macroprotodon* has apparently been in neighbouring areas of Africa for 4–5.5 million years. An alternative possibility is that Iberian *Macroprotodon* arose from accidental human introduction in the last few thousand years. These snakes often live close to human settlements in and around ruins and old buildings and among refuse, which makes accidental transportation more likely (Pleguezuelos and Cardenete, 2002), for instance in consignments of earth, ship ballast or among vegetable products and other cargo. Certainly some snakes have been spread in this way. Another small lizard-eating species, the south Asian *Lycodon capucinus*, has reached some Indian Ocean islands that have never been connected to other land masses. It reached Christmas Island by 1987 (Cogger, 2000), Réunion before 1840 apparently in bales of rice (Cheke, 1987), and Mauritius by about 1870 (A. Bouton, letter in the central archives of Natural History Museum, London). *L. capucinus* may also have reached the Philippines by human agency (Leviton, 1965). The Indian Ocean islands have also received the worm snake *Ramphotyphlops braminus*, and Mauritius has gained a second worm snake, *Typhlops porrectus* (C.G. Jones and E.N. Arnold, personal observations).

It is widely suggested that spread of *Macroprotodon* to the Balearic islands of Menorca and Mallorca was also through human agency (Wade, 1988, 2001; Busack and McCoy, 1990; Pleguezuelos et al., 1994; Pleguezuelos and Cardenete, 2002). These islands were last connected to other land areas during the Messinian salinity crisis 5.6–5.3 million years ago, yet the *M. mauritanicus* found on them show very little difference in their mitochondrial DNA from their continental relatives in Tunisia (and probably also in eastern Algeria; see Fig. 1). Similarity to these populations also extends to many morphological features (Wade, 1988, 2001; Busack and McCoy, 1990). Another possible indication of recent arrival is that there are no fossil *Macroprotodon* known from the Balearics (Pleguezuelos et al., 1994; Alcover et al., 1999), even though the paleontological record of these islands is quite good (Alcover and Mayol, 1981; Quintana, 1998; Alcover et al., 1999). It has been postulated that the populations on Mallorca and Menorca may have been introduced during the second century B.C. (Pleguezuelos and Cardenete, 2002).

Of the *Macroprotodon* populations on small islands off the coast of North Africa, those on Lampedusa and Djerba are of the *M. cucullatus* type, while the ones on Galita, Zembra, and Zembretta are *M. mauritanicus*, exhibiting no morphological differences from Algerian and Tunisian populations. On the basis of their proximity to mainland populations, snakes on Habibas are probably *M. abubakeri.* All these islands are on the African continental shelf and *Macroprotodon* may have reached them during the sea-level falls that accompanied Pleistocene ice ages, to be isolated once the water rose again with the onset of warmer conditions.

Specimens assigned to *M. cucullatus* are known from the Hoggar Mountains of southern Algeria, which contain areas of montane steppe (Papenfuss, 1969) and are cooler and moister than the surrounding desert. They also occur in southern Morocco and coastal Western Sahara where habitats are also more suitable than further inland (see Fig. 1). These populations are probably relicts from periods when relatively more mesic conditions occurred intermittently in the Sahara (Duplessy et al., 1989). Mesic conditions permitted such Sahelian reptiles as *Crocodylus niloticus*, *Dasypeltis scaber*, *Bitis arietans*, and *Lamprophis fulginosus* to penetrate further north into the Sahara and some species now largely confined to the Maghreb to move south into this region including *Coluber algirus* and perhaps the amphibians *Rana saharica* and *Bufo viridis*. Movements of this kind may have been possible as recently as 8000 years ago, when the Sahara was temporarily moist and "scarcely a desert at all" (Roberts, 1989). As the Sahara became progressively more arid again, the populations of *Macroprotodon* and the other species would have been isolated. The degree of genetic divergence, length of separation and taxonomic status of these relict populations will only be elucidated when molecular data are available for them.

Nomenclature. Molecular data make it certain that the nomenclature of *Macroprotodon* will require modification. As animals assigned to *M. cucullatus* do not appear to form a monophyletic group, it may be necessary to restrict the name *cucullatus* to the more eastern specimens closest to the type locality of Lower Egypt. The phylogenetic association of Moroccan specimens assigned to *M. c. textilis* with those of *M. brevis* raises the possibility that all animals in the clade concerned should be called *M. textilis* as this is the older name. The type locality of *textilis* is vague, being cited as "Deserts of West Algeria." Doumergue (1901) thought the holotype was actually from El Aricha (34° 13′N, 1° 16′W) which is quite close to localities of snakes presently assigned to *M. brevis*. However, the collector of the holotype, F. Schousboé, probably travelled widely so there can be no certainty about this informal restriction of the type locality (Wade, 2001). Actual changes in nomenclature should be delayed until further molecular work on all the populations presently assigned to *M. cucullatus* has been carried out.

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