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Evolution of *Mesobuthus gibbosus* (Brullé, 1832) (Scorpiones: Buthidae) in the northeastern Mediterranean region

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Abstract

Sequence data derived from two mitochondrial markers, 16S rRNA and COI genes, were used to infer the evolutionary history of 47 insular and mainland populations covering most of the distributional range of the northeastern Mediterranean scorpion species *Mesobuthus gibbosus*. Based on the estimated divergence times of *Mesobuthus* lineages, the temporal frame of the genus differentiation in the northeastern Mediterranean region is placed in middle Miocene (15 million years ago). The biogeographic affinities of *M. gibbosus* populations point towards a mainly vicariant pattern of differentiation of the species which is consistent with the geological events that transformed the Aegean region during the period from 12 to 5 million years ago. *M. gibbosus* is an old northeastern Mediterranean species that has retained valuable bits of genetic information, reflecting some of the oldest vicariant events that have occurred in the area. Most importantly, the history witnessed by *M. gibbosus* has not been obscured by more recent palaeoevents of the region. Therefore, the case of *M. gibbosus* is in favour of a taxon-oriented 'perception' of the natural history of a given area.

Keywords: 16S rRNA, COI, Mediterranean region, *Mesobuthus*, phylogeography

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Introduction

During the last decade, several studies have made use of molecular data in order to explore the biogeographic affinities that occurr in various parts of the northeastern Mediterranean region. Most of these studies have focused on the Aegean archipelago since it is characterized by high levels of diversity and endemism (Strid 1997; Sfenthourakis & Legakis 2001) and a complex palaeogeographic history (Anastasakis & Dermitzakis 1990). The above-mentioned biogeographic studies have regarded both invertebrate (Douris *et al*. 1995; Gantenbein *et al*. 2000; Gantenbein & Largiadèr 2002; Parmakelis *et al*. 2003, 2005, 2006) and vertebrate (Beerli *et al*. 1996; Weisrock *et al*. 2001; Poulakakis *et al*. 2003, 2005a, b, c; Kasapidis *et al*. 2005) terrestrial taxa, as well as plants (Bittkau & Comes 2005). Although these studies have contributed significantly to the resolution of the evolution in this area, there is a lot of discrepancy among them. The recorded discrepancies do not necessarily reflect an endogenous inadequacy of the implemented approach — although this could be true for some of the studies — but rather derive from the taxon-orientated 'perception' of the evolutionary history of the area. This 'perception' depends both on biological attributes of the relevant taxon and on the time of its differentiation in the region. Consequently, far from having gathered most of the missing pieces of the puzzle, we still need more studies on various taxonomic groups with common biological attributes and a more or less similar natural history.

As pointed out by Gantenbein & Largiadèr (2002) and Parmakelis *et al*. (2006), the scorpion genera distributed in the northeastern Mediterranean region may serve the purpose of elucidating the complex biogeographic affinities occurring in the area very well. For example, the possibility of a phylogenetic clade to be shaped not by the most recent palaeovents occurring in the region but by older ones has

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been shown for the scorpion *Iurus dufoureius* (Parmakelis *et al*. 2006).

The most widely distributed buthid species in the area of concern is *Mesobuthus gibbosus* (Brullé, 1832) (Fet *et al*. 2000b). The family Buthidae is distributed all over the world (except Antarctica and New Zealand), especially the tropics, subtropics and part of the temperate zone (Fet *et al*. 2000b). However, in the northern part of the eastern Mediterranean, only the genus *Mesobuthus* (Vachon, 1950) can be found, with *M. gibbosus* being the only *Mesobuthus* species present in the Balkans. *M. gibbosus* is known to occur in Albania, Montenegro, Former Yugoslav Republic of Macedonia and Greece (Fet *et al*. 2000b). Besides the Balkan Peninsula it can also be found in Turkey. Until recently, the species was thought to be distributed in Syria, Israel and Lebanon (Fet *et al*. 2000b) as well. However, recent findings (Fet *et al*. 2000a) have clarified that the *Mesobuthus* of Israel, Lebanon and presumably of Syria, is *M. nigrocinctus* (Ehrenberg, 1828), a species that was considered for a long time synonymous to *M. gibbosus*. Quite recently the *Mesobuthus* species of the island of Cyprus was considered to be different from *M. gibbosus* and a new species, *M. cyprius*, was described (Gantenbein *et al*. 2000).

The use of molecular (mtDNA) markers in the study of *M. gibbosus* allowed us to infer the phylogeography of *M. gibbosus* populations. Based on the phylogenetic pattern obtained and using a well-dated palaeoevent of the study region, we estimated the divergence times of lineages. Using these estimates, we addressed the following major questions: (i) What is the time frame of differentiation of the species in the study area? (ii) Which are the most influential processes (vicariance or dispersal) shaping the distributional pattern of *M. gibbosus* lineages in the region? (iii) Is the distribution pattern of *M. gibbosus* lineages compatible with the palaeogeographic history of the study region? (vi) How congruent is the sequence of events being witnessed by *M. gibbosus* with that 'perceived' by other taxa?

Materials and methods

Ingroup taxa

In all the analyses we used samples of *Mesobuthus gibbosus* from 29 islands and islets of the Aegean Archipelago, one locality from the island of Kerkyra (Ionian Sea), seven localities from mainland Greece, and six localities from Turkey (Fig. 1, Table 1). In mainland areas and islands with large surface (i.e. Crete, Rhodes), we included more than one sampling localities. Most of the specimens were newly collected for this study, while some sequences were retrieved from genetic databases. Details on origin of samples and accession numbers of sequences produced or retrieved are given in Table 1. All specimens and DNA extracts produced for this study have been deposited in the Natural History Museum of Crete (NHMC).

Outgroup taxa

The choice of the outgroup taxa in a phylogenetic analysis is far from trivial. Aiming at obtaining a well-resolved phylogeny we used several outgroup species whose sequences were either obtained from genetic databases or determined for this study. We included three additional *Mesobuthus* species. Furthermore, preliminary phylogenetic analyses indicated that the most appropriate distant outgroups were the buthid taxa *Androctonus australis* (Linnaeus, 1758), *Buthus occitanus* (Amoreux, 1789) and *Buthus mardochei* Simon, 1878. These species have repeatedly been used in studies presenting well-resolved buthid phylogenies (Gantenbein & Largiadèr 2003; Gantenbein *et al*. 2003). Therefore, their selection as outgroups in the present analysis is further validated.

Specimen preservation, DNA extraction, amplification and sequence determination

All the individuals (specimens) used in the study were placed in absolute ethanol immediately after collection, while ethanol was also injected into each specimen's body with the use of a syringe.

DNA extraction was carried out as described in Parmakelis *et al*. (2005b). DNA was extracted from a total of 37 *M. gibbosus* and one *M. cyprius* specimens (Table 1). All sequences produced for this study have been deposited in GenBank under Accession nos DQ310811–DQ310884 (Table 1).

Fragments of two mitochondrial genes (16S rRNA and cytochrome oxidase subunit I) were PCR amplified. For the amplification of the 16S rRNA gene, we targeted an approximately 380-bp-long fragment and used a pair of scorpion-specific primers. The sequences of the respective primers are reported in Gantenbein *et al*. (1999). For the amplification of the COI gene, we initially used the universal primers LCO1490 and HCO2198 (targeting a 708-bp fragment) as reported in Folmer *et al*. (1994).A new scorpion specific (forward) primer was subsequently designed from the sequences obtained using the universal primers. The sequence of the primer was: 5′-GCCDGGWTCTTT-BATTGGGGA-3′. The PCR conditions were the same for both the COI and 16S rRNA genes and are described by Parmakelis *et al*. (2005b). Automated sequencing of the forward strand of each mitochondrial DNA (mtDNA) gene was performed in a PE-ABI377 sequencer (using BigDye terminator chemistry). The primers in the sequencing reactions were the same as in the amplification procedure.

Sequences alignment and genetic data analyses

The same type of analysis was applied in both sets (16S rRNA, COI) of sequences. Multiple sequence alignments were done with CLUSTAL W (Thompson *et al*. 1994) of

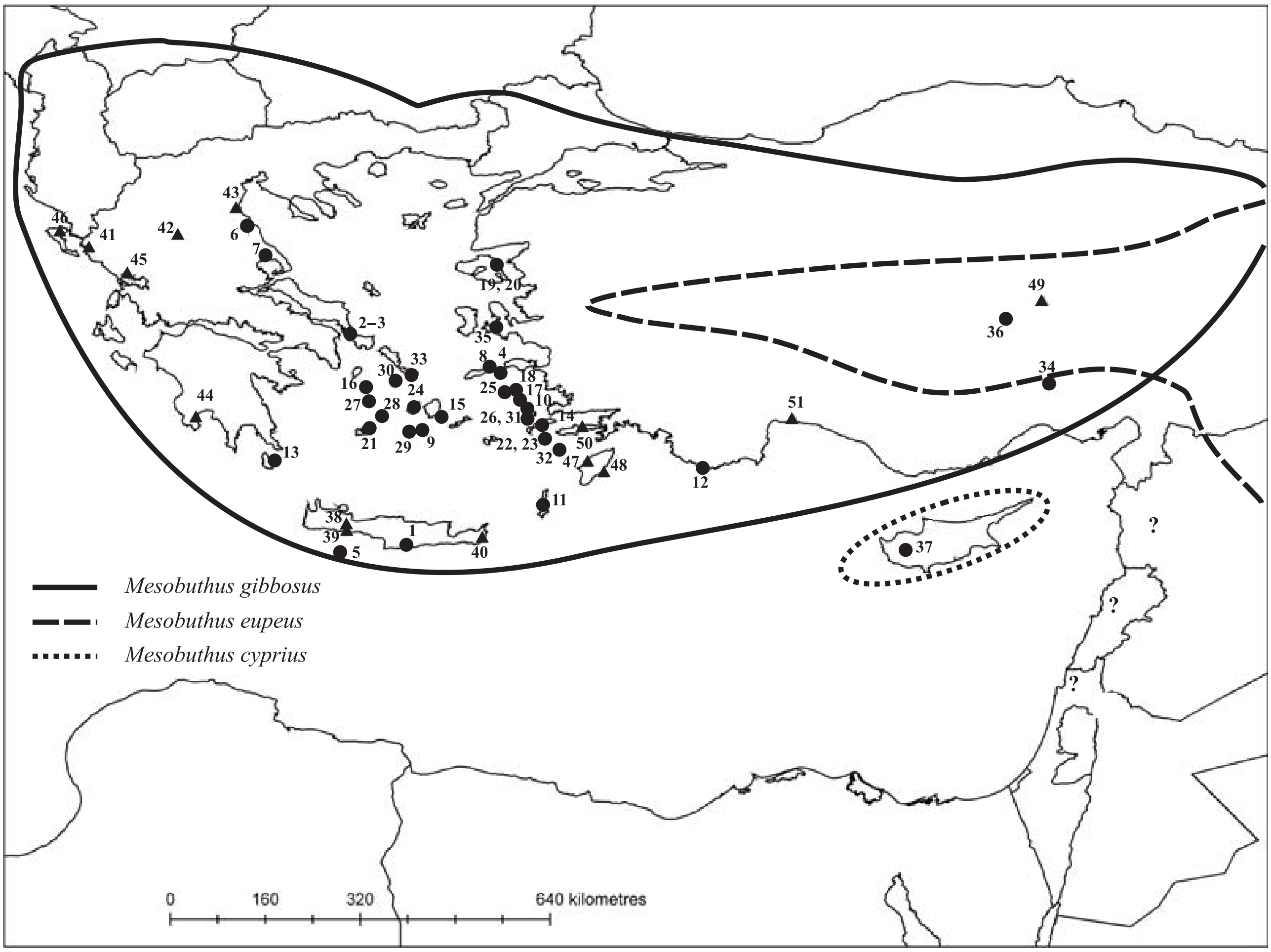

Fig. 1 Distribution maps of *Mesobuthus gibbosus*, *M. cyprius* and *M. eupeus* and locations of sampled populations (●) of *M. gibbosus* and *M. cyprius*. The distribution maps have been drawn following Fet *et al.* (2000b), Gantenbein *et al.* (2000), Crucitti & Cicuzza (2001), Karatas & Karatas (2001) and Karatas & Karatas (2003). Triangles (▲) denote origin of specimens (sequences) retrieved from genetic data banks. Question marks (?) are unverified references of *M. gibbosus*.

Table 1 List of the specimens (in alphabetical order) of *Mesobuthus gibbosus* and outgroups used in the study, map codes of sampled specimens, species names, geographic origin, and accession numbers of sequences

Map code	Specimens	Species/ subspecies	Geographic origin	Accession nos (16S-COI)
Sequences produced for this study				
1	CR Kofinas	*M. gibbosus*	Crete Island, Asterousia Mt., Kofinas peak	DQ310811–DQ310848
2	Evvoia1	*M. gibbosus*	Evvoia Island, Nea Styra	DQ310813–DQ310850
3	Evvoia2	*M. gibbosus*	Evvoia Island, Nea Styra	DQ310814–DQ310851
4	Fournoi	*M. gibbosus*	Fournoi islet, Bali beach SW	DQ310815–DQ310852
5	Gavdos	*M. gibbosus*	Gavdos Island, Lavrakas beach	DQ310816–DQ310853
6	GR Ossa1	*M. gibbosus*	Mainland Greece, Ossa Mt., Spilia — Ampelakia	DQ310817–DQ310854
7	GR Volos	*M. gibbosus*	Mainland Greece, Volos	DQ310818–DQ310855
8	Ikaria	*M. gibbosus*	Ikaria Island, Kerame	DQ310819–DQ310856
9	Ios	*M. gibbosus*	Ios Island, Chora to Psathi, 6 Km before Psathi	DQ310820–DQ310857
10	Kalymnos	*M. gibbosus*	Kalymnos Island, Myrties to Arginouda	DQ310821–DQ310858
11	Karpathos	*M. gibbosus*	Karpathos Island, Volada — Lasto, 2.5 Km after the junction	DQ310822–DQ310859
12	Kastelorizo	*M. gibbosus*	Kastelorizo Island, Kastelorizo village on the path to Agios Georgios church	DQ310823–DQ310860

Table 1 *Continued*

Map code	Specimens	Species/ subspecies	Geographic origin	Accession nos (16S-COI)
13	Kythira	*M. gibbosus*	Kythira Island, Agia Moni	DQ310824–DQ310861
14	Kos	*M. gibbosus*	Kos Island, Kardamaina beach	DQ310825–DQ310862
15	Koufonisi	*M. gibbosus*	Koufonisi Islet, Agios Georgios to Plateia Pounta	DQ310826–DQ310863
16	Kythnos	*M. gibbosus*	Kythnos Island, Liotrivi	DQ310827–DQ310864
17	Leipsoi	*M. gibbosus*	Leipsoi Island, Leipsoi to Platy Gialo	DQ310828–DQ310865
18	Leros	*M. gibbosus*	Leros Island, Kamara to Tourloti	DQ310829–DQ310866
19	Lesvos1	*M. gibbosus*	Lesvos Island, Mystegna 1 Km NE	DQ310830–DQ310867
20	Lesvos2	*M. gibbosus*	Lesvos Island, Mystegna 1 Km NE	DQ310831–DQ310868
21	Milos	*M. gibbosus*	Milos Island, Treia Pigadia beach	DQ310832–DQ310869
22	Nisyros1	*M. gibbosus*	Nisyros Island, Nikia to Avlaki	DQ310833–DQ310870
23	Nisyros2	*M. gibbosus*	Nisyros Island, Kaldera	DQ310834–DQ310871
24	Paros	*M. gibbosus*	Paros Island, Marathi, ancient quarries	DQ310835–DQ310872
25	Patmos	*M. gibbosus*	Patmos Island, Chora to Kouvari, next to Profitis Ilias	DQ310836–DQ310873
26	Safonidi	*M. gibbosus*	Safonidi Islet	DQ310837–DQ310874
27	Serifos	*M. gibbosus*	Serifos Island, Plakes, Agios Georgios	DQ310838–DQ310875
28	Sifnos	*M. gibbosus*	Sifnos Island, Vathy, before the junction to Fykiada	DQ310839–DQ310876
29	Sikinos	*M. gibbosus*	Sikinos Island, Chorio	DQ310840–DQ310877
30	Syros	*M. gibbosus*	Syros Island, Pyrgos O.T.E. Antenna	DQ310841–DQ310878
31	Telendos	*M. gibbosus*	Telendos islet	DQ310842–DQ310879
32	Tilos	*M. gibbosus*	Tilos Island, Leivadia W	DQ310843–DQ310880
33	Tinos	*M. gibbosus*	Tinos Island, Chora E	DQ310844–DQ310881
34	Turkey Adana	*M. gibbosus*	Mainland Turkey, Adana, Pozanti Alpuk	DQ310845–DQ310882
35	Turkey Izmir	*M. gibbosus*	Mainland Turkey, Izmir, Alacati 5 km E	DQ310846–DQ310883
36	Turkey Nidge	*M. gibbosus*	Mainland Turkey, Nigde, Amas Yolu	DQ310847–DQ310884
37	Outgroup	*M. cyprius*	Cyprus Island, Roudia bridge	DQ310812–DQ310849
Ingroup and outgroup sequences retrieved from genetic data banks				
38	CR Kares	*M. gibbosus*	Crete Island, Kares	AJ402576–AJ783466
39	CR Vraskas	*M. gibbosus*	Crete Island, Vraskas	AJ402577–AJ783467
40	CR Zakros	*M. gibbosus*	Crete Island, Zakros	AJ402574–AJ783464
41	GR Igoumenitsa	*M. gibbosus*	Mainland Greece, Igoumenitsa	AJ550691–AJ550711
42	GR Kalampaka	*M. gibbosus*	Mainland Greece, Kalampaka	AJ783458–AJ783472
43	GR Litochoro	*M. gibbosus*	Mainland Greece, Litochoro	AJ368239–AJ783471
44	GR Mathia	*M. gibbosus*	Mainland Greece, Mathia	AJ402571–AJ550710
45	GR Vigla	*M. gibbosus*	Mainland Greece, Vigla	AJ402573–AJ783470
46	Kerkyra	*M. gibbosus*	Kerkyra Island, Petalia	AJ783454–AJ783463
47	Rodos1	*M. gibbosus*	Rhodes Island, Kritinia	AJ402555–AJ783492
48	Rodos2	*M. gibbosus*	Rhodes Island, Lindos	AJ402550–AJ783473
49	Turkey Avanos	*M. gibbosus*	Mainland Turkey, Avanos	AJ402587–AJ550712
50	Turkey Datca	*M. gibbosus*	Mainland Turkey, Datca	AJ402585–AJ783496
51	Turkey Selale	*M. gibbosus*	Mainland Turkey, Selale	AJ402580–AJ783494
	Outgroup	*M. cyprius*	Cyprus Island, Diorios (Tepebasi)	AJ550680–AJ550698
	Outgroup	*M. cyprius*	Cyprus Island, Kantara	AJ550681–AJ550699
	Outgroup	*M. caucasicus*	Kazakhstan	AJ550674–AJ550692
	Outgroup	*M. eupeus eupeus*	Mainland Turkey, Gulsehir	AJ550688–AJ550701
	Outgroup	*M. eupeus mongolicus*	China	AJ550682–AJ550700
	Outgroup	*M. eupeus thersites*	Kazakhstan	AY228141–AJ550704
	Outgroup	*A. australis*	Tunisia, Nefta	AJ506868–AJ506919
	Outgroup	*B. mardochei*	Morocco, Ras el Ma	AJ506854–AJ506903
	Outgroup	*B. occitanus*	France, Narbonne	AJ514322–AJ506907

the ClustalW Service at the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw) using the default parameters. The computer-generated alignment was further adjusted manually. Additionally, the alignment of the COI data set was checked against COI sequences of *M. gibbosus* available in genetic data banks. The number of transitions (ti) and transversions (tv) occurring among each pairwise combination of individual sequences (including outgroups) were plotted against pairwise (p) genetic distances to evaluate possible mutational saturation (following Lydeard *et al.*

1996). Signs of saturation were evident in both mtDNA gene data sets. In the 16S rRNA data set, the transitions exhibited homoplasy, whilst in the COI data set the transversions exhibited some level of saturation.

Pairwise genetic distances were estimated using MEGA (Kumar *et al*. 2001) and the Kimura 2-parameter model (Kimura 1980).

Phylogenetic analyses

Analyses of phylogenetic inference were conducted using two methods: maximum parsimony (MP) and Bayesian inference (BI). Nucleotides were used as discrete, unordered characters. To examine whether the sequences from the two mtDNA genes could be combined in a single analysis, a partition-homogeneity test (Farris *et al*. 1995) was run in PAUP* (Swofford 2002) and significance was estimated with 1000 repartitions. This test did not indicate any conflicting phylogenetic signals between the data sets ($P = 0.67$) and, given that the mtDNA genes are linked, data sets from both genes were used together in all phylogenetic analyses.

MP analysis was performed in PAUP* (Swofford 2002), with heuristic searches using stepwise addition and performing tree-bisection–reconnection (TBR) branch swapping (Swofford *et al*. 1996). A weighted parsimony was also performed. The weighting scheme was based on the saturation exhibited by the two mtDNA genes. Consequently, the transversions were weighted twice as transitions in the 16S rRNA data set, and vice versa in the COI data set. In all MP analysis confidence in the nodes was evaluated by 1000 bootstrap replicates (Felsenstein 1985).

A BI was performed with the program MRBAYES 3.1 (Ronquist & Huelsenbeck 2003) using the partitioned data set and applying in each gene partition the parameters of the substitution model suggested by MODELTEST 3.7 (Posada & Crandall 1998) according to the Akaike Information Criterion (AIC; Akaike 1974). The number of generations was set to 10^6. The average standard deviation of split frequencies of the two simultaneous and independent runs performed by MRBAYES 3.1 was used to determine the stationarity point of likelihoods (see MRBAYES 3.1 manual). According to this index, stationarity was achieved well before 2×10^5 generations. A tree was sampled every 100th generation and, consequently, the summaries of the BI relied on 20 000 samples (from 2 runs). From each run 7501 samples were used, while 2499 were discarded as burn-in phase. From the remaining 15 002 trees, a consensus tree was constructed. Support of the nodes was assessed with the posterior probabilities of reconstructed clades as estimated by MRBAYES3.1 (Ronquist & Huelsenbeck 2003).

The posterior probability of alternative topologies was assessed by filtering (in PAUP*; Swofford 2002) the Bayesian trees found (after stationarity was achieved), using as constrain the tree with the topology to be evaluated.

Molecular clock and estimation of divergence times

A likelihood ratio test (LRT) on the presence of a molecular clock was performed according to Huelsenbeck & Crandall (1997). The LRT was negative (LRT = 135, d.f. = 58, $P << 0.05$), therefore a clocklike evolution of the involved sequences could not be assumed. Consequently, we estimated divergence times by relaxing the molecular clock assumption. This was accomplished through the implementation of the nonparametric rate smoothing (NPRS) method of Sanderson (1997) and the Powell algorithm in the program R8S version 1.7 (Sanderson 2003). NPRS (Sanderson 1997) relaxes the assumption of a molecular clock by using a least squares smoothing of local estimates of substitution rates. It estimates divergence times for all unfixed nodes and uses as optimality criterion the sum of squared differences in local rate estimates from branch to neighbouring branch.

One of the most valid geotectonic events of the Aegean is the formation of the mid-Aegean trench (12–9 million years ago; Creutzburg 1963; Dermitzakis 1990a). This event has been used and verified in a series of biogeographic studies of vertebrate (Beerli *et al*. 1996; Poulakakis *et al*. 2003; Kasapidis *et al*. 2005), invertebrate (Sfenthourakis 1996; Fattorini 2002; Chatzimanolis *et al*. 2003; Parmakelis *et al*. 2005, 2006) and plant taxa (Bittkau & Comes 2005) of the Aegean Archipelago. We used this event to calibrate the respective node in the phylogenetic tree. The mean divergence times and confidence intervals for each of the remaining nodes in the phylogenetic tree were obtained following the suggestions described in the R8S manual (http://ginger.ucdavis.edu/r8s), using 100 bootstrapped phylograms produced under the MP criterion using the same parameters as in the original MP analysis. The fidelity, with which the applied method (NPRS) explained the branch length variation, was explored using the cross-validation option (Sanderson 2002).

Results

In total, for both mtDNA genes, we sequenced 37 specimens (individuals) of *Mesobuthus gibbosus* and one specimen of *M. cyprius*, while the sequences of 23 specimens (including outgroups) were retrieved from genetic databases (Table 1). The final concatenated alignment of the 16S rRNA and COI genes of *M. gibbosus* and the outgroup species consisted of 762 nucleotides (16S rRNA: 355 bp; COI: 407 bp), all of which were analysed. Of these, 390 (51.2%) were constant and 251 (36.9%) were parsimony informative. For the 16S rRNA gene, ingroup sequence divergence ranged from 0.3% to 14.9%, while for the COI gene, sequence divergence ranged from 0% to 13.3%. The genetic distance (net between group average) as calculated by MEGA (Kumar *et al*. 2001) between *M. gibbosus* and the other *Mesobuthus* species studied ranged from 4.2% (*M. cyprius*) to 12.3% (*M. caucasicus*).

Table 2 Kimura 2-parameter (Kimura 1980) distances (net between group average: MEGA) between *Mesobuthus gibbosus* clades and *Mesobuthus* species

	1	2	3	4	5	6
(1) Clade A3.2						
(2) Clade A3.1	0.04					
(3) Clade A2	0.05	0.05				
(4) Clade A1	0.06	0.05	0.07			
(5) *M. cyprius*	0.06	0.05	0.07	0.07		
(6) *M. eupeus*	0.12	0.12	0.11	0.13	0.13	
(7) *M. caucasicus*	0.14	0.14	0.15	0.15	0.14	0.10

The best-fit model selected by MODELTEST 3.7 (Posada & Crandall 1998) for the 16S rRNA gene was the TrN + Γ (base frequencies: A = 0.3467, C = 0.1588, G = 0.0962, T = 0.3983; shape parameter a = 0.4616). Accordingly, the best-fit model selected for the COI gene was the GTR + I + Γ (base frequencies: A = 0.2055, C = 0.1277, G = 0.2557, T = 0.4111; shape parameter a = 1.1018 and pinv = 0.3659). The BI resulted in a tree (–Ln = 6267.4; Fig. 2) with a topology that was not in total agreement with that of the weighted MP analysis (tree not shown).

The BI (Fig. 2) revealed three main *M. gibbosus* clades (denoted as A1, A2 and A3). Clade A1 comprises the haplotypes from all the eastern Aegean islands of the study (except Lesvos), along with the haplotype from Datca in southwest Turkey. Clade A2 hosts the haplotypes from the southern islands of the Kyklades plateau (Milos, Ios, Sikinos, Sifnos and Koufonisi). Finally, clade A3 contains the haplotypes from Lesvos, the remaining islands of the Kyklades plateau, the islands of Kerkyra and Kythira, the haplotypes from mainland Greece and the remaining samples from mainland Turkey. A subclade (A3.1) appears within the A3 clade and includes the haplotypes from Crete, Gavdos, Kastelorizo and the haplotype from Selale in southern Turkey. Furthermore, *M. gibbosus* appears as a monophyletic clade, well separated from all outgroup species (Fig. 2).

The MP analysis with the gaps treated as missing and all characters equally weighted, produced 4232 equally parsimonious trees with a length of 785 steps. The weighted MP produced 2500 equally parsimonious trees with a length of 1627 steps. The two analyses were congruent regarding the branching order of the deeper clades, while all the equally parsimonious solutions were the result of terminal branch swapping. However, the statistical support of all major clades was stronger in the weighted MP. The trees produced by the unweighted and weighted MP analyses (trees not shown) have one major topological difference from that of the BI analysis. More specifically, there is a mutual replacement of clades A2 and A3.1. However, the MP topology was weakly supported and the filtering of the Bayesian trees with this topology produced a very low posterior probability (9.5%).

The divergence times (± standard deviations) of the respective nodes are presented in Fig. 2. The genetic distances between the major *M. gibbosus* clades and between *Mesobuthus* species included in the analyses are presented in Table 2.

Discussion

Phylogenetic analyses

An interesting feature of the tree presented in Fig. 2 is the poor resolution of certain clades. As supported by Rokas *et al.* (2002) in a study of gallwasps, the poor resolution of the produced phylogenetic trees may be explained in two ways. First, the topologies might represent actual events (i.e. adaptive radiation). If the rate of speciation for a given window of time is relatively high, inter-lineage genetic differentiation is expected to be low. This would result in a poor resolution of the phylogeny, despite the fact that it is an accurate depiction of the historical relationships between the taxa. Alternatively, the lack of resolution might be due to inadequate data (characters and/or taxa). In a more recent paper, Rokas & Carroll (2005) conclude that phylogenetic accuracy is positively affected mainly by increasing the number of genes. Gantenbein & Keightley (2004) investigated the molecular evolution of nuclear genes in the genus *Mesobuthus* in the east Mediterranean region. They used a small subset (10 islands and 10 mainland populations) of the samples included in our study, and sequenced nine nuclear genetic markers (3856 bp). Despite the huge amount of sequence data involved in their study, the phylogenetic tree produced by the concatenated data set was also weakly supported (bootstrap support below 65%), while the inferred topology was almost identical to the one presented in Fig. 2. Therefore, we are confident that the low resolution of our tree is an accurate depiction of the historical relationships of the taxa involved in the analysis, despite the low statistical support of some major clades.

Gantenbein & Keightley (2004) were the first to present the biogeographic affinities of a limited number of insular and mainland populations of *Mesobuthus gibbosus* within the northeastern Mediterranean. However, the authors have misinterpreted the geological history of the region and used as calibration points separation times that are not validated by any study. For example, they placed the separation time of Crete from Cyprus at 5.33 million years ago (Ma), during the Messinian salinity crisis. This event was imposed by the mid-Aegean trench much earlier during Miocene (Creutzburg 1963; Dermitzakis 1990a). If they had taken that into consideration, most of the estimated divergence times in their study would largely conform to the ones obtained herein.

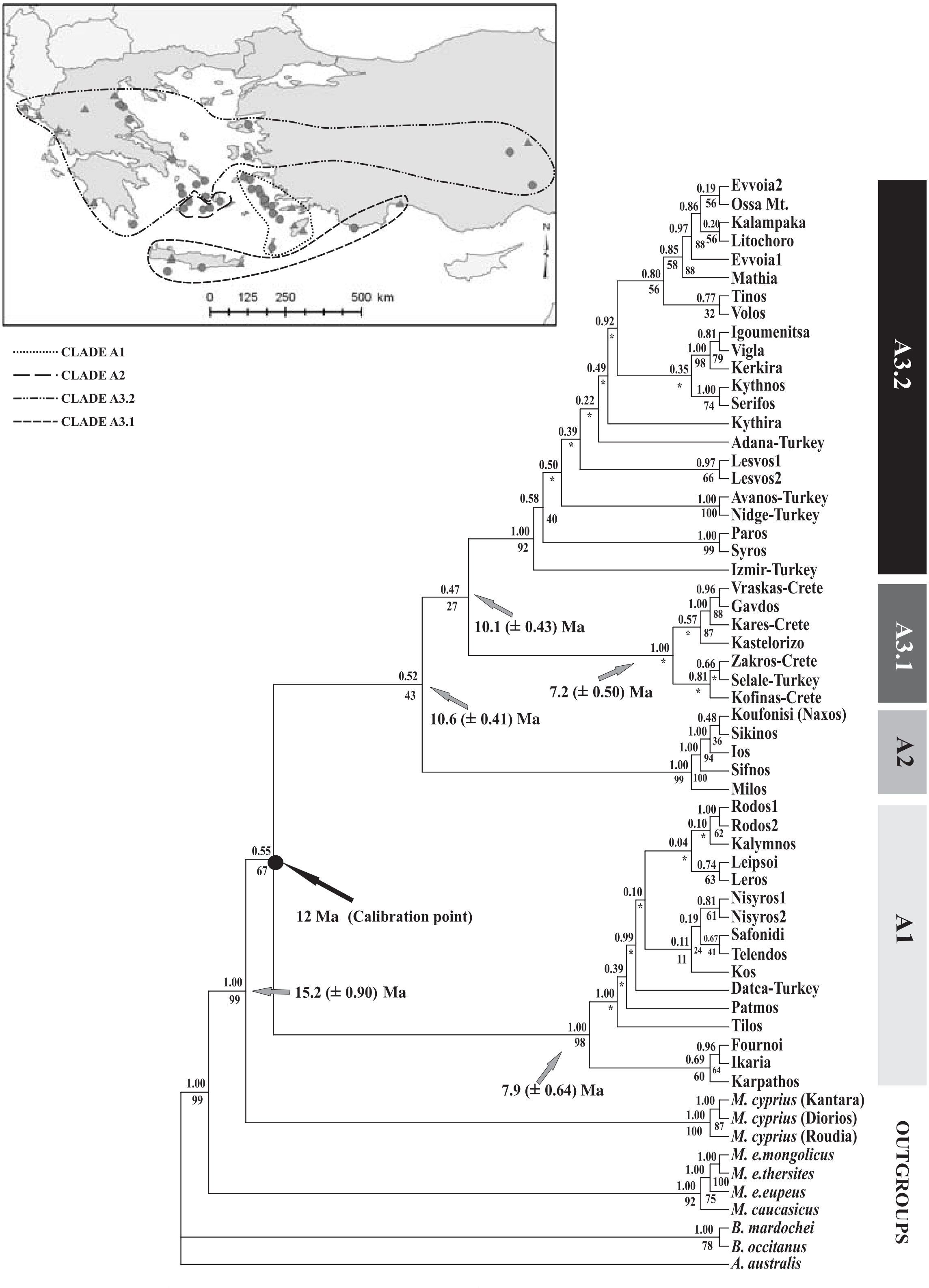

Fig. 2 Bayesian Inference (BI) tree (50% majority rule consensus tree). Numbers below branches indicate the nonparametric bootstrap support values for the nodes in the maximum-parsimony (MP) analyses (based on 1000 replicates). Numbers above branches are the posterior probabilities of the nodes in the BI analysis. Asterisks (*) indicate topology not supported in the MP analysis. The estimated separation times (± standard deviations) for selected nodes (indicated by the grey arrows) are also displayed. The black arrow points at the node used as calibration point. On the embedded map, the distributions of the inferred phylogenetic clades are depicted.

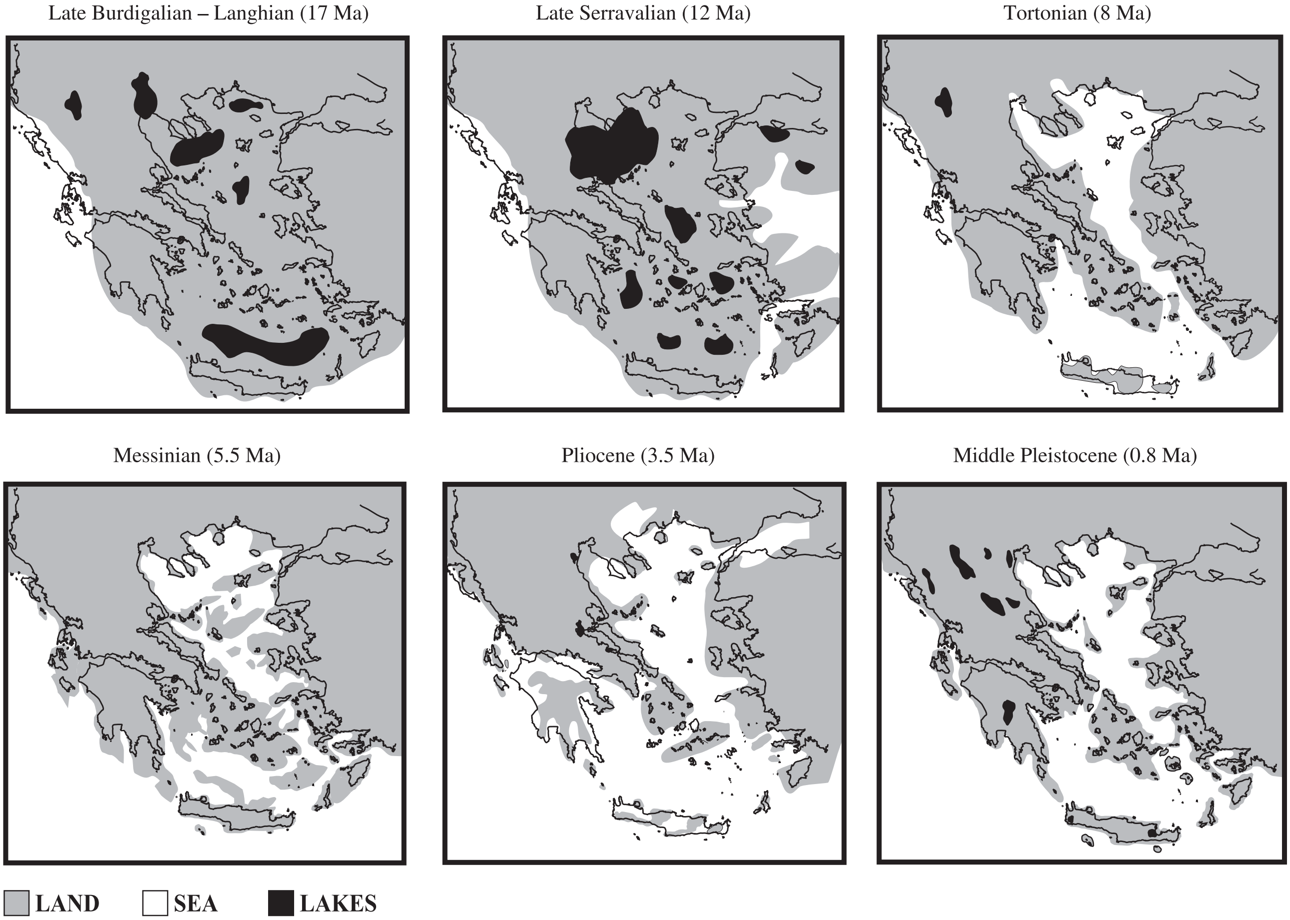

Fig. 3 Greece from Miocene to present (redrawn after Creutzburg 1963; Dermitzakis & Papanikolaou 1981; Dermitzakis 1990a). The maps are drawn based on present geography.

Palaeogeography of the region

During the upper and middle Miocene (23–12 Ma), the Aegean region was part of a united landmass (Agäis) (Dermitzakis & Papanikolaou 1981). The modern history of the Aegean region and the surrounding areas was initiated in the late Seravallian to early Tortonian (12–8 Ma) by tectonic events. Tectonic plated displacements resulted in the break-up of the southern Aegean landmass. Some major events of the palaeogeographic evolution of the Aegean region from late Burdigallian to middle Pleistocene are described in Fig. 3.

Time frame of differentiation

Phylogenetic analyses indicate the existence of three *Mesobuthus gibbosus* clades (Fig. 2). A strong correspondence between clades and geographic origin of the specimens is evident in the phylogeny.

The clustering of the islands and mainland populations of *M. gibbosus* presented in Fig. 2 points towards a mainly vicariant pattern of differentiation of the species in the study area. Additionally, the obtained phylogeographic pattern, along with the estimated divergence times of the major clades, set out the temporal frame of the genus divergence in the study area somewhere in middle Miocene (15 Ma).

Phylogeographic pattern compatibility with the evolutionary history of other taxa and the palaeogeography of the region

The island of Cyprus presumably arose from the floor of the ancient Tethys Sea and from marine sediments, while being the highest elevation emerging from a system of accretionary wedges located south of Crete and extending from the southernmost tip of Italy to the Gulf of Iskenderun (Schüle 2000). According to Dermitzakis (1990b), it is generally assumed that Cyprus was never connected to the mainland, but others (Hadjisterkotis *et al.* 2000) support that it was probably connected to Anatolia during the Messinian salinity crisis. However, in this study, as well as in the study of Gantenbein & Keightley (2004), a direct relation of lineages of Cyprus with Anatolia is not observed. Nevertheless, a connection between Syria and Cyprus has been suggested in the phylogeographic study of the lizard genus *Ablepharus* (Poulakakis *et al*. 2005a). The genetic distances reported in the study of *Ablepharus* indicate a separation time of Cyprus and Syria dating back to 20–11.6 Ma. This time period fits quite well with the estimated divergence time of *Mesobuthus cyprius* in the current study (15.3 Ma). The inclusion of material from Syria could shed more light in the puzzling origin of *M. cyprius*.

Concentrating on the Aegean archipelago and *M. gibbosus*, the divergence times reported in this study are consistent with most of the palaeogeographic events mentioned above. *M. gibbosus* evolved in the region responding to the geological events that transformed the Aegean region during the period from 12 to 5 Ma. Going back in time and according to the phylogeographic pattern and the divergence times displayed in Fig. 2, the formation of the mid-Aegean trench (9–12 Ma: calibration point) forces the split of the *M. gibbosus* populations of the region into two major groups: the west and the east. These groups correspond to clades A1 (east group) and A2-A3 (west group) of the phylogenetic tree of Fig. 2. Focusing on clade A2 that comprises some islands (Milos, Sifnos, Sikinos, Ios and Koufonisi / Naxos) of the southern Kyklades, it can be seen that approximately 10.6 Ma, a probable but unverified geological event resulted in its separation from clade A3. At this point, it is worth mentioning that some of the islands of clade A2 host endemic forms of both vertebrate (Milos Island, *Macrovipera schweizeri*, Herrmann *et al*. 1992; *Podarcis milensis*, Poulakakis *et al*. 2003) and invertebrate taxa (Sifnos Island, *Zonites siphnicus*, Mylonas 1982). Especially for *P. milensis* from Milos it has been reported that an isolated population of *P. taurica* used to have a wider distribution in the past, but was restricted to that island around 6.6 Ma (Poulakakis *et al*. 2005b). The presence of endemic taxa on these islands is a strong indication of the idiosyncratic nature of their palaeogeography. Moreover, the case of *P. milensis* highlights the fact that the separation of the southern Kyklades islands from the northern Kyklades plateau — that occurred 3.5 Ma (see Fig. 3 and Anastasakis & Dermitzakis 1990) and had a significant impact on the present-day distribution of certain Aegean taxa (see Kasapidis *et al*. 2005) — is not the only factor defining the biogeographic patterns observed in these islands today. In addition, a similar pattern of isolation and independent evolution of the island of Naxos relatively to the remaining islands of the Kyklades plateau is corroborated in the study of the plant species *Nigella arvensis* (Bittkau & Comes 2005), even though these authors place the isolation event during Pleistocene.

The separation of subclade A3.2 from A3.1 is estimated at 10.1 Ma (Fig. 2). This is consistent with the isolation of the island of Crete from the Kyklades plateau and mainland Greece, an event that is placed between 9.7 and 8.9 Ma (Creutzburg 1963; Meulenkamp 1985; Peters 1985; Dermitzakis 1990a). This very old separation event has been recorded previously only in the phylogeographic studies of the gecko *Cyrtopodion kotschyi* (Kasapidis *et al*. 2005) and the land snail genus *Mastus* (Parmakelis *et al*. 2005). Clade A3.2 is composed of lineages originating from different parts of the northeastern Mediterranean region. It comprises lineages from the Kyklades plateau (Paros, Syros, Tinos, Kythnos and Serifos), mainland Greece and neighbouring islands (Evvoia Island, Kythira Island, Ossa Mountain, Litochoro, Kalampaka, Igoumenitsa, Vigla and Kerkyra Island), one island of the eastern Aegean islands (Lesvos) along with northern and central populations of mainland Turkey (Adana, Nidge, Avanos and Izmir). The radiation of this clade is estimated to have started approximately 8 Ma and to have lasted until 5 Ma (Fig. 2). The relations of the Greek mainland and island populations are more or less anticipated. However, the links of central and northern Anatolian lineages with regions of the Kyklades plateau and mainland Greece imply that these regions maintained some connections during that time. The formation of the mid-Aegean trench lasted approximately 3 Myr and was progressing northwards. Therefore, it is expected that as the trench gradually expanded northwards, the southern Aegean islands were the first to be affected, while the separation of the northern islands occurred later on. A similar phylogenetic pattern, bringing together Anatolian lineages with lineages from mainland Greece and Kyklades, has been observed in *Cyrtopodion kotschyi* (Kasapidis *et al*. 2005) and the estimated period of occurrence is placed at approximately 6 Ma.

Approximately 7.2 Ma, *Mesobuthus gibbosus* populations of the island of Crete experienced some radiation and different lineages emerged within the island (clade A3.1, Fig. 2). The time frame of radiation of lineages on the island of Crete fits very well with the vicariant events that were governing the geological evolution of the island and resulted in its fragmentation into smaller islands separated by sea (Fig. 3). Furthermore, the temporal frame of this radiation event is in congruence with that estimated for the land snail species of the genus *Mastus* (Parmakelis *et al*. 2005a) from the island of Crete. In the study of *Mastus*, the authors conclude that the radiation events of the Cretan *Mastus* lineages took place 9.3–6.9 Ma. The relationships of the Cretan *Mastus gibbosus* lineages with lineages of south Turkey (Selale) and the island of Kastelorizo seem quite peculiar. It is tempting to speculate that these relationships are the reflection of past connections of Crete with Anatolia dating back to middle or upper Miocene. However the mean genetic distance (Kimura 1980) separating Kastelorizo Island from the Cretan haplotypes is 4% (Kastelorizo-Crete), while the respective value for the Turkish (Selale–Crete) haplotype is 3.9%. The low level of sequence divergence between these areas is a strong indication of a more recent long-distance dispersal. Similar cases of long dispersal between southwest Anatolia and satellite islands of the island of Crete have been recorded in the case of the reptile species *Cyrtopodion kotschyi* (Kasapidis *et al*. 2005). When considering the island of Gavdos, it can be seen that this island exhibits strong relationships with southwest Crete. Gavdos re-emerged from the sea during Pliocene or early Pleistocene without ever being connected to Crete (Fassoulas, personal communication), and was colonized mainly by organisms of the neighbouring Crete. The colonization of Gavdos from Cretan *Mesobuthus gibbosus* lineages some time during Pleistocene is verified in this study (Fig. 2).

Clade A1 consists of all islands located to the east of the mid-Aegean trench along with a population from mainland southwest Turkey (Datca). The relations of the Greek islands within this clade do not deviate from what is expected when considering the fact that these islands have a more or less common history. Investigating the pattern observed in clade A1, we can comment on the fact that the clade that hosts the island of Karpathos is estimated to have become separated at 7.9 Ma (Fig. 2). This estimated time frame is consistent with the isolation of Karpathos (see Fig. 3) at 8 Ma as reported in Dermitzakis & Papanikolaou (1981). However, despite the unresolved relation of Karpathos to the islands of Ikaria and Fournoi (Fig. 2), this is not surprising. Unexpected biogeographic relationships for the island of Karpathos have been reported before (Gantenbein & Largiadèr 2002; Veith & Steinfartz 2004; Bittkau & Comes 2005; Parmakelis *et al*. 2006). Therefore, we concur with Parmakelis *et al*. (2006) who concluded that the relationships of the island of Karpathos do not reflect the most recent vicariant events of the corresponding geographic region, but older ones. Hence, more detailed palaeogeographic and phylogeographic data from a diverse array of organisms distributed on the island of Karpathos and the nearby islands are needed to explain these odd biogeographic affinities. Furthermore, we have to assume that the lineages of the Aegean islands of clade A1 are more widely distributed in the nearby coasts of Turkey.

The scattering of the Anatolian lineages throughout the phylogenetic tree of *M. gibbosus* and the genetic distances separating the mainland populations of Turkey (mean genetic distance within Turkey, d = 6.2%) indicate that *M. gibbosus* of the Turkish mainland should not be considered to be evolving as a single (monophyletic) entity under any circumstances. This validates the hypothesis that the genus *Mesobuthus* is of Central Asian origin, where it is most diverse and where several species and subspecies have been described. These conclusions have already been drawn and justified accordingly in the work of Gantenbein & Keightley (2004).

Concluding remarks

The genus *Mesobuthus* has been differentiating in the northeastern Mediterranean region for approximately 15 Myr. The biogeographic affinities of *Mesobuthus gibbosus* populations point towards a mainly vicariant pattern of differentiation of the species which is consistent with the geological events that transformed the Aegean region during the period from 12 to 5 Ma. *M. gibbosus* is an old northeastern Mediterranean species that has retained valuable bits of genetic information, reflecting some of the oldest vicariant events that have occurred in the area. Most importantly, the history witnessed by *M. gibbosus* has not been obscured by more recent palaeoevents of the region. Therefore, the case of *M. gibbosus* is in favour of a taxon-orientated 'perception' of the evolutionary history of a given area, in the sense that each taxon reveals its own responses to geological and environmental changes.

The sequence divergence (concatenated data set, net between group average) exceeds 5% (Table 2) in the pairwise comparison of clade A1 and the remaining *M. gibbosus* clades in Fig. 2. This level of sequence divergence is similar to the one separating *M. cyprius* and *M. gibbosus* (Table 2). Therefore, it would be justified to consider one of the respective clades as a different *Mesobuthus* species. However, we suggest that a detailed investigation of diagnostic morphological characters that might be able to discriminate between the respective phylogenetic clades is needed before one can assign any clade to species status.

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