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Phylogenetic relationships of *Mesobuthus eupeus* (C.L. Koch, 1839) inferred from *COI* sequences (Scorpiones: Buthidae)

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In this study, the first molecular phylogenetic analysis of *Mesobuthus eupeus* in Iran is presented based on sequence data of a ~ 700-base-pair fragment of cytochrome C oxidase, subunit I. Phylogenetic relationships were inferred using parsimony, maximum likelihood and Bayesian inference. The results support monophyly of *M. eupeus*, but there is a clear divergence between northern and southern clades. The northern clade includes four subspecies – *M. e. eupeus*, *M. e. philippovitschi*, *M. e. afghanus* and *M. e. thersites*; whereas the southern clade is comprised of two others – *M. e. phillipsi* and *M. e. kirmanensis*. Accordingly, possible scenarios for the evolution and phylogeographic structure of *M. eupeus* based on the geological history of the Iranian Plateau were proposed. The observation of two distinct lineages supports the proposal that *M. eupeus* might be a species complex composed of species with highly similar morphological features.

Keywords: *Mesobuthus eupeus*; scorpions; Buthidae; subspecies; cytochrome C oxidase subunit I; Iran

Introduction

Scorpions possess several characteristics notably different from other arthropods. Based on some traits including longevity, age to maturity, complex courtship behaviour, viviparous embryonic development, maternal care, post-embryonic development, low metabolic rate and a degree of social behaviour, they are more similar to long-lived vertebrates (Polis and Sissom 1990; Gantenbein and Largiadèr 2002; Lourenço 2000). Scorpions are traditionally considered to be living fossils and are known for being morphologically highly conserved animals (Sissom 1990). Although these unusual features have been well known for some time, biochemical and molecular methods have only recently been applied to this taxon. DNA sequence evidence was first presented by Gantenbein et al. (1999) for assessing the phylogeny of the genus *Euscorpius*. The phylogeographical studies on euscorpiids (Gantenbein et al. 1997, 1999, 2000; Gantenbein and Scholl 1998; Gantenbein, Fet and Barker 2001; Huber et al. 2001; Fet et al. 2003; Salomone et al. 2007) and buthids (Gantenbein et al. 1999, 2000, 2003, 2005; Gantenbein, Soleglad and Fet 2001; Gantenbein and Keightley 2004; Gantenbein and Largiadèr 2002, 2003; Parmakelis et al. 2006) using

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nuclear and mitochondrial markers revealed that many previously described species are in fact constituted by sibling species not easily differentiable based on morphological features. In some instances extensive genetic divergence had evolved in the absence of a significant morphological differentiation. For example, on the basis of molecular evidence, *Euscorpius (Euscorpius) carpathicus* (Linnaeus 1767) is now considered a complex of at least seven sibling species (Gantenbein and Largiadèr 2002; Vignoli et al. 2005; Salomone et al. 2007).

Scorpions of the genus *Mesobuthus* Vachon, 1950 represent a useful terrestrial model for the study of molecular evolution (Gantenbein and Keightley 2004). This genus has recently been the subject of molecular studies for intra- and interspecific phylogeographic and phylogenetic studies (Gantenbein et al. 2003; Gantenbein and Keightley 2004; Parmakelis et al. 2006). The genus *Mesobuthus* was erected when Vachon (1950) initiated the revision and splitting of the traditional genus *Buthus* Leach, 1815. The taxonomic composition of this genus is still controversial and there is no consensus on the number of species comprising the genus *Mesobuthus*. Some described species may in fact be composed of sibling species, and still novel species are being described. For instance, separate species such as *Mesobuthus cyprius* from Cyprus (Gantenbein et al. 2000) and *Mesobuthus songi* from China (Lourenço et al. 2005) were described. In addition, *Mesobuthus nigrocinctus* has been recorded from Israel and Turkey (Fet et al. 2000; Teruel 2000; Karataş and Karataş 2001; 2003). It is widely accepted that the genus *Mesobuthus* includes at least 12 species (Fet and Lowe 2000; Gantenbein et al. 2000). The modern distribution of the genus *Mesobuthus* extends from the Balkans through China, and its occurrence in northern parts of central Asia represents the northern limit of scorpion distribution in Asia (Fet 1994; Gromov 2001).

The scorpion *Mesobuthus eupeus* (C. L. Koch, 1839) is the type species of the genus *Mesobuthus*. This species is the most widely dispersed species of the genus *Mesobuthus* and one of the most dispersed members of the family Buthidae. It occurs in eastern and central parts of Turkey, Armenia, Azerbaijan, Georgia, southern Russia, Syria, Iraq, Iran, Afghanistan, Pakistan, Central Asia, southern Mongolia and northern China (Birula 1917; Farzanpay 1987; Vachon and Kinzelbach 1987; Fet 1989; Kovařík 1997; Fet et al. 2000; Gromov 2001; Shi et al. 2007). This extensive geographic distribution is accompanied by morphological variations on the basis of which several subspecies have been described (Pocock 1889; Birula 1900, 1905, 1917; Vachon 1952). As early as 1917 Birula grouped the described subspecies of *M. eupeus* into two 'sections' or species groups, *M. eupeus* and *M. thersites*. Moreover, he recognized three "natio" within the nominotypical subspecies, *M. e. eupeus*. Among the described subspecies, 14 are considered formally valid (Fet and Lowe 2000). But the taxonomic status and relationships between subspecies of *M. eupeus* has not been recently examined and revisions may be appropriate. The morphological characteristics used for determination and assessing the relationships of these subspecies are inconclusive and vague (Birula 1900, 1905). With respect to the *M. eupeus* subspecies recorded from Iran, authors are in disagreement (Farzanpay 1987; Fet 1989) and some authors believe that *M. eupeus* is a species complex (Gantenbein et al. 2003).

Mitochondrial DNA markers can be used for resolving taxonomic ambiguities in *M. eupeus* by a molecular approach. Mitochondrial DNA sequences are used in the present study with the objective of describing the evolutionary lineages of this species. Additionally, the times of divergence between lineages are estimated. Finally, possible processes that shaped the current distribution of *M. eupeus* in the Iranian Plateau are discussed.

Materials and methods

Sample collection

According to Prendini (2001, 2005) and Lamoral (1979), members of the genus *Mesobuthus* are habitat generalist lapidicolous scorpions that shelter under stones or any other available covers. Here, most specimens were collected at daytime by rock rolling in the field and a few were caught at night using the ultraviolet light detection method (Lowe et al. 2003). A portable ultraviolet flashlight equipped with inidium gallium nitride light-emitting diodes was used for specimen collection at night. After collection, ethanol was injected into the specimens' bodies and they were preserved in 70–96% ethanol. Geographic coordinates of most collection sites were recorded using a hand held global positioning system (Garmin$^{\mathrm{TM}}$). Geographic coordinates of a small number of sites were assessed by reference to the gazetteers and official topographic maps of Iran.

A distribution map of collection sites was created using ArcView GIS 3.1 (Environmental System Research Institute, Redlands, CA, USA), by superimposing locality records on layers depicting political boundaries and topography. The topographic contour layer was based on the GTOPO30 raster grid coverage, available on the U.S. Government Public Information Exchange Resource at http://edc.usgs.gov/products/elevation/gtopo30.

Ingroup and outgroup taxa

Fifty-nine adult specimens of *M. eupeus* (Figure 1, Table 1) were collected from different localities in Iran. Additionally, 31 sequences were retrieved from GenBank (Table 1). Details of the origin of samples and the accession numbers of sequences used in this research or retrieved from databases are given in Table 1. All vouchers and DNA extracts have been deposited in the Zoological Museum, University of Tehran.

Three buthid species, *Androctonus australis* (Linnaeus 1758), *Buthus occitanus* (Amoreux 1789) and *Buthus mardochei* Simon, 1878 are frequently used as outgroups in buthid phylogenetic studies (Gantenbein and Largiadèr 2003; Gantenbein et al. 2003; Parmakelis et al. 2006). The species closest to these species that is found in Iran is the Old World buthid, *Androctonus crassicauda* and it was used as outgroup in the present study. Additionally, sequences of three other *Mesobuthus* species, *M. gibbosus*, *M. cyprius* and *M. caucasicus* were included (Gantenbein and Keightley 2004; Gantenbein et al. 2005).

Molecular laboratory methods

DNA was isolated from the specimens, using the GenNetBio$^{\mathrm{TM}}$ genomic DNA Extraction kit following the manufacturer's instructions (Seoul, South Korea). Polymerase chain reaction (PCR) was subsequently performed to amplify a fragment of approximately 700 base pairs (bp) of the mitochondrial cytochrome C oxidase, subunit I (*COI*) gene. Primers used were LCO1490: 5′-GGTCAACAAATCATCATA AAGATATTGG-3′ (Folmer et al. 1994) and Nancy: 5′-CCCGGTAAAATTAAA

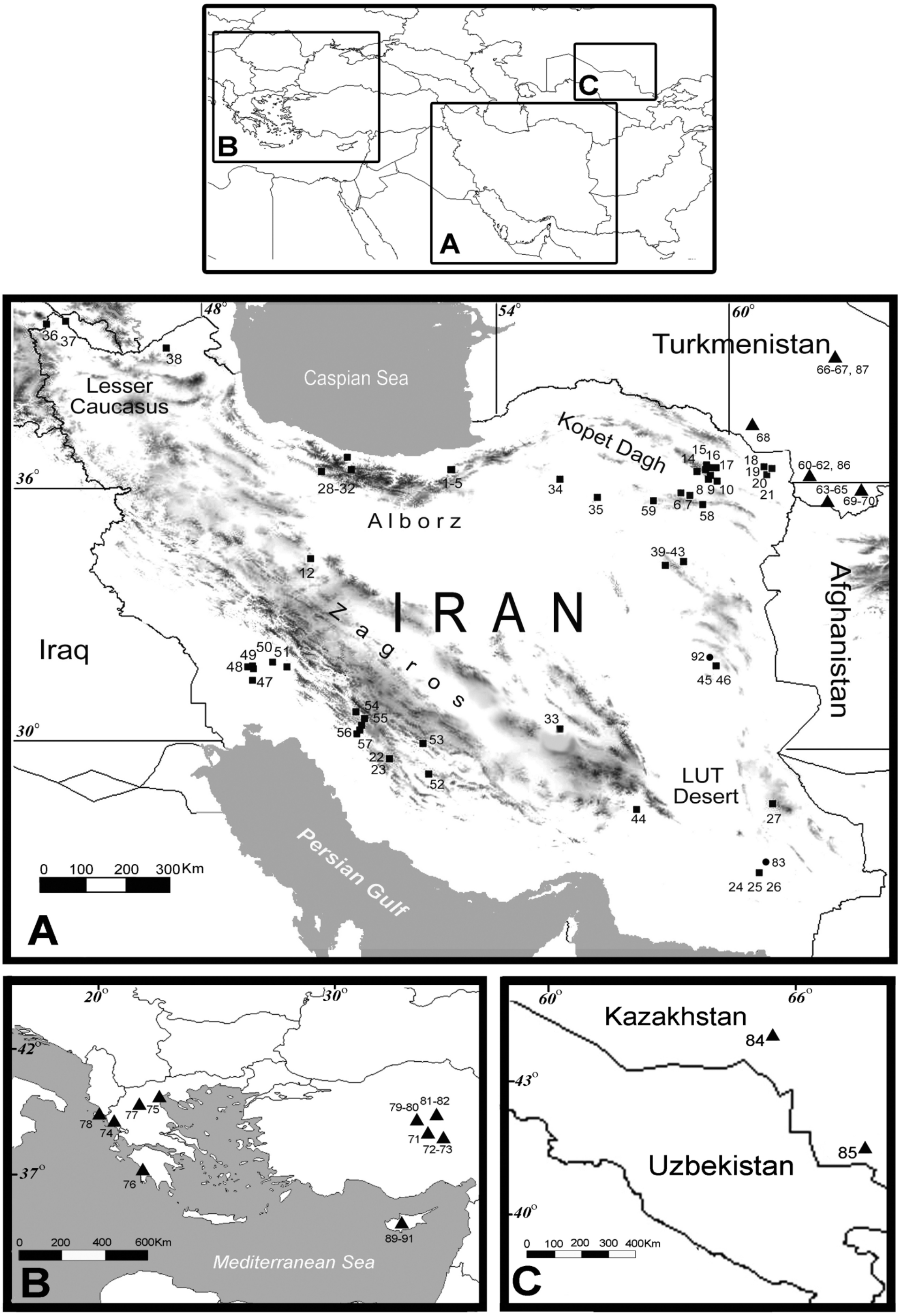

Figure 1. Collection sites of *Mesobuthus eupeus* (■). Triangles (▲) shown in (A), (B) and (C) denote origin of sequences of *Mesobuthus* species retrieved from GenBank. Names of collection sites are given in Table 1. Localities of two of the outgroup specimens used in the study are also shown in (A; ●).

Table 1. *Mesobuthus eupeus* specimens and outgroups used in the present study.

Map code*	Specimen†	Species	Museum code	Geographic location	Accession numbers of *COI* sequences†
1	Me MZ1099	*M. eupeus*	ZUTC-arach-1099	Kiasar – Mazandaran province	HM567346
2	Me MZ1083	*M. eupeus*	ZUTC-arach-1083	Kiasar – Mazandaran province	HM567348
3	Me MZ1085	*M. eupeus*	ZUTC-arach-1085	Kiasar – Mazandaran province	HM567349
4	Me MZ1086	*M. eupeus*	ZUTC-arach-1086	Kiasar – Mazandaran province	HM567350
5	Me MZ1092	*M. eupeus*	ZUTC-arach-1092	Kiasar – Mazandaran province	HM567347
6	Me RK1056	*M. eupeus*	ZUTC-arach-1056	Neishabour – Pivezhan	HM567364
7	Me RK1057	*M. eupeus*	ZUTC-arach-1057	Neishabour – Pivezhan	HM567366
8	Me RK1011	*M. eupeus*	ZUTC-arach-1011	Mashhad – Moghan village	HM567362
9	Me RK1015	*M. eupeus*	ZUTC-arach-1015	Mashhad – Moghan village	HM567361
10	Me RK1016	*M. eupeus*	ZUTC-arach-1016	Mashhad – Moghan village	HM567363
11	Me RK1055	*M. eupeus*	ZUTC-arach-1055	Neishabour – Pivezhan	HM567367
12	Me NI1112	*M. eupeus*	ZUTC-arach-1112	Esphehan province – Niasar	HM567380
13	Me RK1040	*M. eupeus*	ZUTC-arach-1040	ca. 10 km south of Mashhad	HM567358
14	Me RK1051	*M. eupeus*	ZUTC-arach-1051	ca. 10 km south of Mashhad	HM567358
15	Me RK1053	*M. eupeus*	ZUTC-arach-1053	ca. 10 km south of Mashhad	HM567359
16	Me RK1054	*M. eupeus*	ZUTC-arach-1054	ca. 10 km south of Mashhad	HM567360
17	Me RK1008	*M. eupeus*	ZUTC-arach-1008	5 km south of Mashhad (northeast Iran)	HM567357
18	Me RK1032	*M. eupeus*	ZUTC-arach-1032	Sarakhs road – Robate-Sharaf	HM567375
19	Me RK1039	*M. eupeus*	ZUTC-arach-1039	Sarakhs road – Robate-Sharaf	HM567377
20	Me RK1033	*M. eupeus*	ZUTC-arach-1033	Sarakhs road – Robate-Sharaf	HM567372
21	Me RK1035	*M. eupeus*	ZUTC-arach-1035	Sarakhs road – Robate-Sharaf	HM567374
22	Me FA1123	*M. eupeus*	ZUTC-arach-1123	Kenar tapeh – Kazeroon road	HM567338
23	Me FA1124	*M. eupeus*	ZUTC-arach-1124	Kenar tapeh – Kazeroon road	HM567339
24	Me SB1132	*M. eupeus*	ZUTC-arach-1132	Baluchistan – Bampur	HM567381
25	Me SB1133	*M. eupeus*	ZUTC-arach-1133	Baluchistan – Bampur	HM567368

(*Continued*)

Table 1. (Continued).

Map code*	Specimen†	Species	Museum code	Geographic location	Accession numbers of *COI* sequences†
26	Me SB1134	*M. eupeus*	ZUTC-arach-1134	Baluchistan – Bampur	HM567382
27	Me SB1135	*M. eupeus*	ZUTC-arach-1135	Baluchistan – south of Zahedan	HM567369
28	Me MZ1003	*M. eupeus*	ZUTC-arach-1003	Southwest Chalus – Kordichal	HM567352
29	Me MZ1004	*M. eupeus*	ZUTC-arach-1004	Southwest Chalus – Kordichal	HM567353
30	Me MZ1005	*M. eupeus*	ZUTC-arach-1005	Southwest Chalus – Kordichal	HM567354
31	Me MZ1006	*M. eupeus*	ZUTC-arach-1006	Southwest Chalus – Kordichal	HM567355
32	Me MZ1007	*M. eupeus*	ZUTC-arach-1007	Southwest Chalus – Kordichal	HM567356
33	Me YZ1138	*M. eupeus*	ZUTC-arach-1138	Yazd-Mehriz-Marvast road	HM567370
34	Me SE1130	*M. eupeus*	ZUTC-arach-1130	Semnan – Biarjomand	HM567373
35	Me SE1131	*M. eupeus*	ZUTC-arach-1131	Semnan – Mohammad Abad	HM567351
36	Me AZ1118	*M. eupeus*	ZUTC-arach-1118	Bazargan – Gerik road	HM567335
37	Me AZ1119	*M. eupeus*	ZUTC-arach-1119	Bazargan, Oskanlu – Kalibar road	HM567337
38	Me AZ1120	*M. eupeus*	ZUTC-arach-1120	Buralan – Poldasht road	HM567336
39	Me RK1001	*M. eupeus*	ZUTC-arach-1001	Khorasan: Gonabad – 10 km northeast Kakhk	HM567371
40	Me RK1145	*M. eupeus*	ZUTC-arach-1145	Khorasan: Gonabad – 10 km northeast Kakhk	HM567390
41	Me RK1146	*M. eupeus*	ZUTC-arach-1146	Khorasan: Gonabad – 6 km south Kakhk	HM567391
42	Me RK1147	*M. eupeus*	ZUTC-arach-1147	Khorasan: Gonabad – 6 km south Kakhk	HM567392
43	Me RK1148	*M. eupeus*	ZUTC-arach-1148	Khorasan: Gonabad – 6 km south Kakhk	HM567393
44	Me KR1158	*M. eupeus*	ZUTC-arach-1158	Jiroft – Khatoon Abad village. (southern Iran)	HM567383
45	Me SK1106	*M. eupeus*	ZUTC-arach-1106	Khorasan: Nehbandan – Mighan village	HM567378
46	Me SK1107	*M. eupeus*	ZUTC-arach-1107	Khorasan: Nehbandan – Mighan village	HM567379
47	Me KH1160	*M. eupeus*	ZUTC-arach-1160	Khuzestan province – Bagh Malek	HM567385
48	Me KH1161	*M. eupeus*	ZUTC-arach-1161	Khuzestan province – Bagh Malek	HM567386
49	Me KH1162	*M. eupeus*	ZUTC-arach-1162	Khuzestan province – Bagh Malek	HM567387
50	Me KH1163	*M. eupeus*	ZUTC-arach-1163	Khuzestan province – Bagh Malek	HM567388

51	Me KH1164	*M. eupeus*	ZUTC-arach-1164	Khuzestan province – Bagh Malek	HM567389
52	Me FA843	*M. eupeus*	ZUTC-arach-843	Fars province – Kazeroon – Borazjan road	HM567340
53	Me FA851	*M. eupeus*	ZUTC-arach-851	Fars province – Firouz Abad	HM567341
54	Me FA8632	*M. eupeus*	ZUTC-arach-8632	Fars province – Ghir-o-Karzin road	HM567343
55	Me FA8693	*M. eupeus*	ZUTC-arach-8693	Fars province – Ghir-o-Karzin road	HM567345
56	Me FA8691	*M. eupeus*	ZUTC-arach-8691	Fars province – Ghir-o-Karzin road	HM567344
57	Me FA8633	*M. eupeus*	ZUTC-arach-8633	Fars province – Ghir-o-Karzin road	HM567342
58	Me RK1159	*M. eupeus*	ZUTC-arach-1159	Neishabour, Aliabad village	HM567384
59	Me RK1180	*M. eupeus*	ZUTC-arach-1180	Neishabour, northern slope of Binaloud Mt	HM567365
60	Me TUi1	*M. eupeus*	NA	Turkmenistan: Badkhyz Nature Reserve	AJ783586
61	Me TUr1	*M. eupeus*	NA	Turkmenistan: Badkhyz Nature Reserve	AJ783595
62	Me TUs1	*M. eupeus*	NA	Turkmenistan: Badkhyz Nature Reserve	AJ783596
63	Me TUm1	*M. eupeus*	NA	Turkmenistan: northwest Chemenibit	AJ783588
64	Me TUm2	*M. eupeus*	NA	Turkmenistan: northwest Chemenibit	AJ783589
65	Me TUj1	*M. eupeus*	NA	Turkmenistan: northwest Chemenibit	AJ783587
66	Me Tula	*M. eupeus*	NA	Turkmenistan: Repetek, Karakum	AJ550707
67	Me TUa2	*M. eupeus*	NA	Turkmenistan: Repetek, Karakum	AJ783580
68	Me TUn1	*M. eupeus*	NA	Turkmenistan: west Kazarma	AJ783590
69	Me TUp1	*M. eupeus*	NA	Turkmenistan: Kushka River valley	AJ783592
70	Me TUp2	*M. eupeus*	NA	Turkmenistan: Kushka River valley	AJ783593
71	Me TR1	*M. eupeus*	NA	Turkey: Guelsehir	AJ550701
72	Me TR2	*M. eupeus*	NA	Turkey: Cemilkoey	AJ550702
73	Me TR3	*M. eupeus*	NA	Turkey: Cemilkoey	AJ550703
74	MgGRb1	*M. gibbosus*	NA	Greece: Igoumenitsa	AJ550711
75	MgGRd	*M. gibbosus*	NA	Greece: Litochoro	AJ783471
76	MgGRa1	*M. gibbosus*	NA	Greece: Mathia	AJ550710
77	MgGRg	*M. gibbosus*	NA	Greece: Kalampaka	AJ783472
78	MgCOa	*M. gibbosus*	NA	Greece: Petalia	AJ783463
79	MgCAd	*M. gibbosus*	NA	Turkey: Hacibectas	AJ783462
80	MgCAb	*M. gibbosus*	NA	Turkey: Hacibectas	AJ783460
81	MgCAc	*M. gibbosus*	NA	Turkey: Avanos	AJ783461

(*Continued*)

Table 1. (Continued).

Map code*	Specimen†	Species	Museum code	Geographic location	Accession numbers of *COI* sequences†
82	MgCAa	*M. gibbosus*	NA	Turkey: Avanos	AJ783459
83	Mc1136	*M. caucasicus*	ZUTC-arach-1136	Iran: Sistan and Baluchistan- Bampur	HM567334
84	McKZb1	*M. caucasicus*	NA	Kazakhstan: northwest of Baigakum	AJ550693
85	Mc KZc1	*M. caucasicus*	NA	Kazakhstan: Kyzylkum Desert	AJ783602
86	Mc TUj2	*M. caucasicus*	NA	Turkmenistan: Badkhyz Nature Reserve	AJ783614
87	Mc TUo1	*M. caucasicus*	NA	Turkmenistan: Repetek Nature Reserve	AJ783515
88	Mc TUi2	*M. caucasicus*	NA	Turkmenistan: Chagaly, Akhal Region	AJ783509
89	Mcy1	*M. cyprius*	NA	Cyprus: Roudia bridge	AJ550698
90	Mcy2	*M. cyprius*	NA	Cyprus: Kantara	AJ550699
91	Mcy3	*M. cyprius*	NA	Cyprus: Tepebasi	DQ310849
92	Ac1110	*Androctonus crassicauda*	ZUTC-arach-1110	Iran: Nehbandan	HM567333

Notes: COI, cytochrome C oxidase, subunit I; NA, not available; ZUTC, Zoological Museum, University of Tehran.
*Map codes correspond to locations shown in Figure 1.
†All non-*Mesobuthus eupeus* (Me) specimens served as outgroups.
‡Obtained from: http://www.ncbi.nlm.nih.gov/

ATATAAACTTC-3′ (Simon et al. 1994). Each PCR contained 2.5 μl 10 × PCR buffer (100 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$, 500 mM KCl), 2 μl dNTP mix (2.5 mM of each dNTP); 0.6 μl (= 6 pmol) of each primer, 0.125 μl *Taq* DNA polymerase (5 units/μl, Takara Bio Inc., Shiga, Japan), and 1–5 μl DNA template. The PCR thermal regimen consisted of one cycle of 2.5 min at 94°C; five cycles of 45 seconds at 94°C, 45 seconds at 45°C, and 1 min at 72°C; 35 cycles of 45 seconds at 94°C, 1 min at 51°C and 1 min at 72°C; and a final cycle of 10 min at 72°C. Sequencing was performed using ABI Big Dye terminator chemistry and an ABI Prism 3700 instrument (Applied Biosystems, Foster City, CA, USA). The sequences were analysed using SEQUENCHER version 4.1.1 (GeneCodes Corporation, Ann Arbor, MI, USA). Sequence qualities were visually checked and the infrequent sequences containing ambiguous callings were not used.

Sequence alignment was performed using CLUSTALX (version 1.8, Thompson et al. 1997) under its default parameters. Validity of callings for variant nucleotides at highly conserved positions was confirmed visually. All novel *COI* sequences were deposited in GenBank [http://www.ncbi.nlm.nih.gov with the accession numbers HM567333 to HM567393].

Estimation of intraspecific and interspecific sequence divergence

DNASP 5 (Rozas et al. 2003) and ARLEQUIN 2.00 (Schneider et al. 2000; Excoffier et al. 2005) software was used to compare sequence characterizations, nucleotide diversities and levels of divergence between *M. eupeus*, *M. caucasicus*, *M. gibbosus* and *M. cyprius* species. Intraspecific sequence divergences among *M. eupeus* sequences were also calculated using MEGA4 (Tamura et al. 2007).

Phylogenetic analyses

Phylogenetic relationships were analysed by maximum parsimony, maximum likelihood and Bayesian inference using PAUP (Swofford 1998) and MRBAYES v.3.1 (Ronquist and Huelsenbeck 2003) software. Parsimony analyses with PAUP (version 4.0) were performed using a heuristic search with equal weighting of all characters, 1000 random stepwise addition and tree bisection–reconnection, (TBR) branch swapping. The robustness of inferred relationships and nodal supports was checked with 1000 bootstrap replications.

For maximum likelihood analyses, the best substitution model was selected with MODELTEST (version 3.7, Posada and Crandall 1998). Parameters from this best model were used for subsequent likelihood analyses in PAUP with 100 random stepwise additions and TBR branch swapping. Finally, to assess the nodal support of the resulting clades, 250 bootstrap pseudoreplicates were performed.

Bayesian analysis, was carried out with MRBAYES (version 3.2, Ronquist and Huelsenbeck 2003) using the same substitution model used for maximum likelihood analysis, this method was recently proposed as a relatively faster method for estimating phylogenetic trees (Holder and Lewis 2003). The Monte Carlo–Markov chain length was 2,500,000 generations and trees were sampled every 100 generations. Bayesian topology and posterior probabilities were computed by majority rule consensus after burning of all pre-asymptotic tree scores.

The Bayesian approach was also used for analysis of alignment partitioned by codon positions. The best substitution models for this purpose were selected using PHYML v.2.4.4 (Guindon and Gascuel 2003). The molecular clock hypothesis (i.e. equal rates across all branches) was tested with a χ^2 approximated likelihood-ratio test using PAUP (Felsenstein 1981; Huelsenbeck and Crandall 1997; Huelsenbeck and Rannala 1997; Swofford et al. 1996). The trees were compared using two degrees of freedom for operational taxonomic units (OTUs − 2) i.e. $92 - 2 = 90$.

Morphological comparisons

To test whether genetic divergence is in line with morphological divergence of *M. eupeus* clades, a comparison of general morphology was performed. A total of 17 morphometric ratios were determined for representatives of each clade. Measurements were taken with a > 0.02-mm accurate graticule on an Olympus BHZ stereomicroscope following Stahnke (1970) and Lamoral (1979). Abbreviations of morphometric ratios used are as follows: Ca_l/aw, carapace length to anterior width; Ca_l/pw, carapace length to posterior width; Ca_aw/pw, carapace anterior width to posterior width; ca_x/y, the distance between anterior margin of carapace and anterior edge of median eyes to the distance between anterior edge of median eyes and posterior margin of carapace; Mt-I_l/w, metasomal segment I length to width; Mt-I_l/h, metasomal segment I length to height; Mt-II_l/w, metasomal segment II length to width; Mt-II_l/h, metasomal segment II length to height; Mt-III_l/w, metasomal segment III length to width; Mt-III_l/h, metasomal segment III length to height; Mt-IV_l/w, metasomal segment IV length to width; Mt-IV_l/h, metasomal segment IV length to height; Mt-V_l/w, metasomal segment V length to width; Mt-V_l/h, metasomal segment V length to height; CH_l/ml, chela length to manus length; Tl_l/w, telson length to width; Tl_L/h, telson length to height.

Principal component and canonical discriminant analyses were applied to *M. eupeus* and *M. caucasicus* specimens collected from different localities of Iran using SPSS ver. 13 and PAST 1.91 (Hammer et al. 2001). Principal component analysis was performed to determine whether any of the geographic populations are morphologically distinct. Also, to show discrimination between populations, canonical discriminant analysis using Mahalanobis distance was performed.

Results

Sequence characteristics and levels of variations

In total, 59 individuals of *M. eupeus*, one specimen of *M. caucasicus* and one specimen of *A. crassicauda* were sequenced. The *COI* sequences belonging to *M. gibbosus* (nine sequences), *M. cyprius* (three sequences) and Central Asian specimens of *M. caucasicus* (five sequences) and *M. eupeus* (14 sequences) were retrieved from GenBank. The average fragment length of sequences we sequenced and those obtained from GenBank were, respectively, 650 bp and 496 bp. Considering all sequences, frequencies of A, G, C and T were, respectively, 19.7%, 26.7%, 12.6% and 41.2%.

Estimates of % variation among sequences were based on a fragment length of 672 nucleotides, which is the longest length used in the alignments. In the alignment of all sequences, including all *Mesobuthus* and outgroup species, 395 (58.3%) positions were completely conserved and 277 (41.7%) positions were variable. One hundred and eighty-three (27.8%) of the positions were parsimony informative. Overall nucleotide diversity (P_i) for the 92 sequences was 0.076. Among the 59 *M. eupeus* specimens, in the 672-bp fragment, 196 (29.16%) variable sites and 476 (70.83%) completely conserved sites were observed. Of the variable sites, 158 bp (23.51%) were parsimony informative. P_i for the 59 organisms was 0.0617. To assess interspecific divergence, genetic distances between *M. eupeus* and three other *Mesobuthus* species were calculated using net between group average using MEGA4. The average distances between *M. eupeus* and *M. caucasicus*, *M. cyprius* and *M. gibbosus* were, respectively, 7.2%, 10% and 8.4%. Uncorrected pairwise Kimura two-parameter (K2P) distance distances for all 92 sequences were also calculated using the maximum composition likelihood method. Values for intraspecific distance within the genus *Mesobuthus* ranged from 0% to 15% and values for interspecific distances ranged from 7% to 20%.

Best-fit nucleotide substitution model

The best-fit model of sequence evolution selected for *COI* determined by MODELTEST 3.7 under Akaike information criterion was GTR + Γ + I (lnL = 5901.9956; Akaike information criterion 11,823.991). Estimates for the model parameters employed in maximum-likelihood searches included estimated base frequencies ($\pi_A = 0.1970$, $\pi_C = 0.1262$, $\pi_G = 0.2656$, $\pi_T = 0.4113$), rate parameter estimates ([A< >C] = 0.1933; [A< >G] = 13.5717; [A< >T] = 1.4370; [C< >G] = 0.5092; [C< >T] = 3.1889; [G< >T] = 1.000), gamma distribution shape parameter ($\alpha = 1.2841$) and proportion of invariant sites (pinvar = 0.5145). This model was subsequently used for Bayesian phylogenetic inference of *COI* sequences. For partitioned sequence data by codon positions, GTR + Γ, F81 and HKY + Γ substitution models were used for first, second and third codon positions, respectively.

The molecular clock hypothesis was tested with the χ^2 approximated likelihood-ratio test with df = 92–2 = 90. This test makes use of parameters described above. The estimated p-value ($2\delta = 2(lnL_0 - lnL_1)$; $lnL_0 = 6008.82$, $lnL_1 = 5901.99$; df = 90, $p = 0.1089$) supported the molecular clock hypothesis.

Phylogenetic analyses

Maximum likelihood, parsimony and Bayesian analyses all produced phylogenetic trees that were almost congruent. All predicted the existence of an Eastern and a Western clade, subclades A and B within the *M. eupeus* clade, and further divisions of subclades A and B as described below. The topology resulting from maximum likelihood analysis is presented in Figure 2. Division into two major *Mesobuthus* clades labelled Eastern and Western is evident. The Western clade is constituted by *M. gibbosus* and *M. cyprius*, whereas the Eastern clade is constituted by *M. eupeus* and *M. caucasicus*. The *M. eupeus* clade itself is divided into subclades A and B, and subclades A and B are each further subdivided, respectively, into clades A_1, A_2 and B_1, B_2. Further subdivision of B_2 itself into $B_{2.1}$ and $B_{2.2}$ may be warranted. A majority rule consensus tree resulted from parsimony analysis (length 1092, Consistency Index (CI): 0.3425; Retention Index (RI): 0.7985; Rescaled Consistency Index (RC): 0.2735 and 183 parsimony informative characters), and was similar in topology to the presented maximum likelihood tree (maximum parsimony tree not shown). In the parsimony

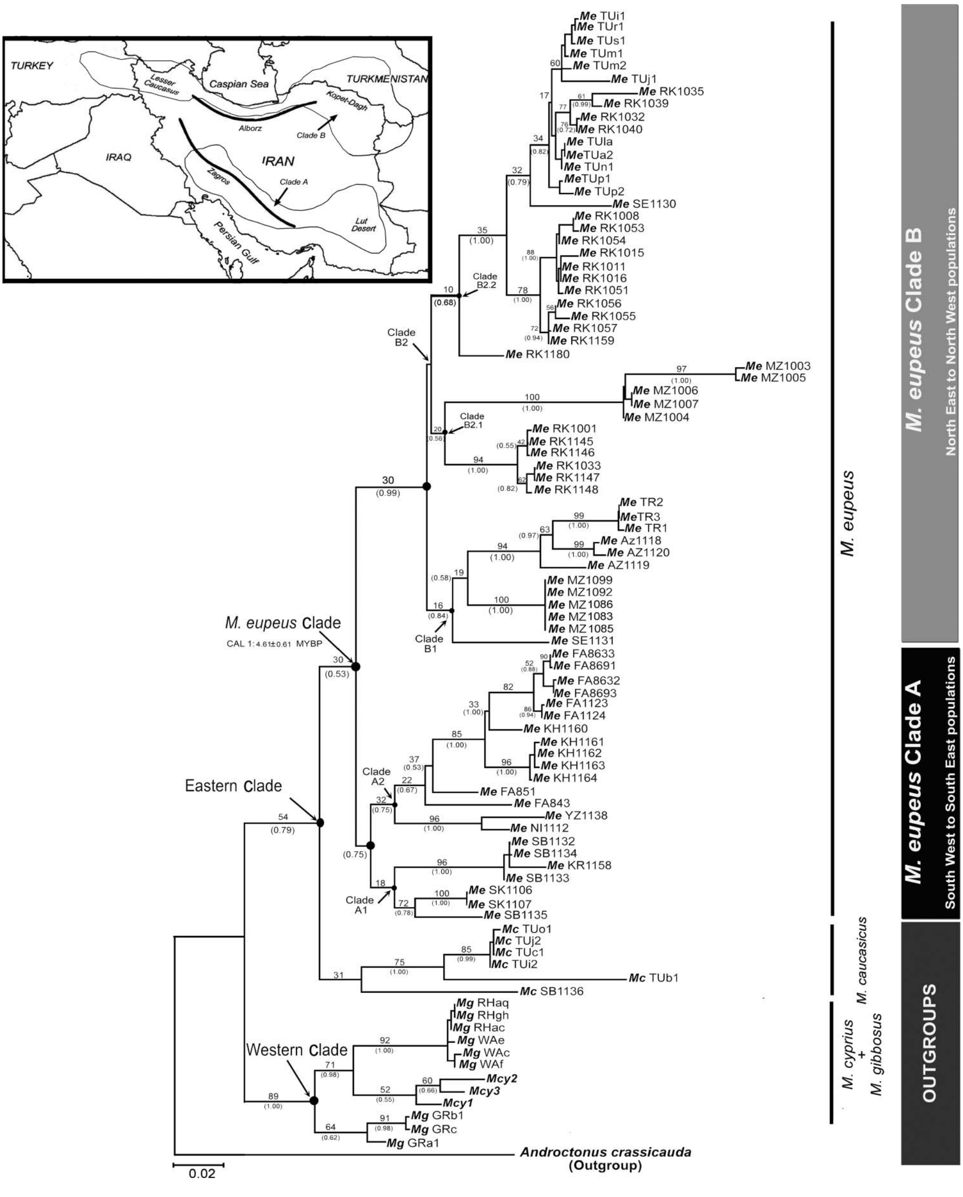

Figure 2. Fifty per cent majority rule consensus tree resulting from maximum likelihood analysis. Numbers below branches indicate the posterior probabilities of the nodes in the Bayesian inference analysis. Numbers above branches are the bootstrap values of the nodes in the maximum likelihood analysis. The estimated separation time for calibration point (± standard deviations) is also displayed. The geographic distributions of inferred clades are shown on the embedded map.

consensus tree, there were no nodes that contradicted those presented in the maximum likelihood tree. Topologies resulting from Bayesian analysis using complete and partitioned sequence data based on codon positions of nucleotides were also similar to topologies described above.

Table 2. K2P genetic distances between *Mesobuthus eupeus* clades, *M. caucasicus*, *M. cyprius*, *M. gibbosus* and *Androctonus crassicauda*.

	A_1	A_2	B_1	$B_{2.1}$	$B_{2.2}$	*M.ca*	*M.cy*	*M.g*
Clade A_1								
Clade A_2	0.05							
Clade B_1	0.05	0.06						
Clade $B_{2.1}$	0.06	0.06	0.05					
Clade $B_{2.2}$	0.06	0.07	0.05	0.04				
M. caucasicus	0.07	0.07	0.07	0.08	0.07			
M. cyprius	0.09	0.10	0.10	0.11	0.10	0.08		
M. gibbosus	0.08	0.08	0.09	0.09	0.08	0.07	0.05	
Outgroup	0.13	0.13	0.13	0.14	0.13	0.13	0.13	0.11

Notes: A_1, A_2, B_1, $B_{2.1}$ and $B_{2.2}$ represent *Mesobuthus eupeus* clades shown in Figure 2. *M.ca*, *Mesobuthus caucasicus*; *M.cy*, *Mesobuthus cyprius*; *M.g*, *Mesobuthus gibbosus*; and Outgroup, *Androctonus crassicauda*
In the analysis, sequences of all specimens of respective clades shown in Figure 2 were used.

Genetic distances and age estimation

Genetic distances between *M. eupeus* clades A_1, A_2, B_1, $B_{2.1}$ and $B_{2.2}$, three *Mesobuthus* species, *M. caucasicus*, *M. gibbosus* and *M. cyprius* and the outgroup species *A. crassicauda* were calculated (Table 2). K2P and net between group averages were used in the calculations. The genetic distances between the *M. eupeus* clades ranged between 4% and 7%, and the maximum distance was observed between clades A_2 and $B_{2.2}$. The distances between *M. eupeus* and other *Mesobuthus* species ranged from 7% to 11%. Distances between the other *Mesobuthus* species were 5% to 8%. Finally, as expected, the distances between *Mesobuthus* species and the outgroup species *A. crassicauda* were in the range of 11% to 14%.

Inspection of the geographic distribution of clades shown in Figure 2 showed that those of clades A and B, respectively, clustered in the southern and northern regions of Iran (Figure 2). The Zagros Mountains separating these two regions may well serve as a geographic barrier. To test the role of Zagros formation in the separation of these clades, the age of calibration point that represents the node at which clades A and B were separated, was estimated (Figure 2). The mean divergence between the clades A and B was 6.00 ± 0.8% (mean ± SE). For estimation of divergence time, calculations were performed assuming divergence rates of 1.3% and 1.9% per million years. The published divergence rates of *COI* for Arthropoda ranged between these figures. The estimated divergence times between clades A and B based on the 1.3% and 1.9% values were, respectively 4.61 ± 0.61 million years ago (mya) and 3.15 ± 0.42 mya.

Morphological comparisons

Principal component analysis based on 17 morphological ratios showed a deep divergence between *M. eupeus* and *M. caucasicus*, as expected (Figure 3). The first three components resulting from the analysis explained 45.15%, 16.88% and 15.64% of the

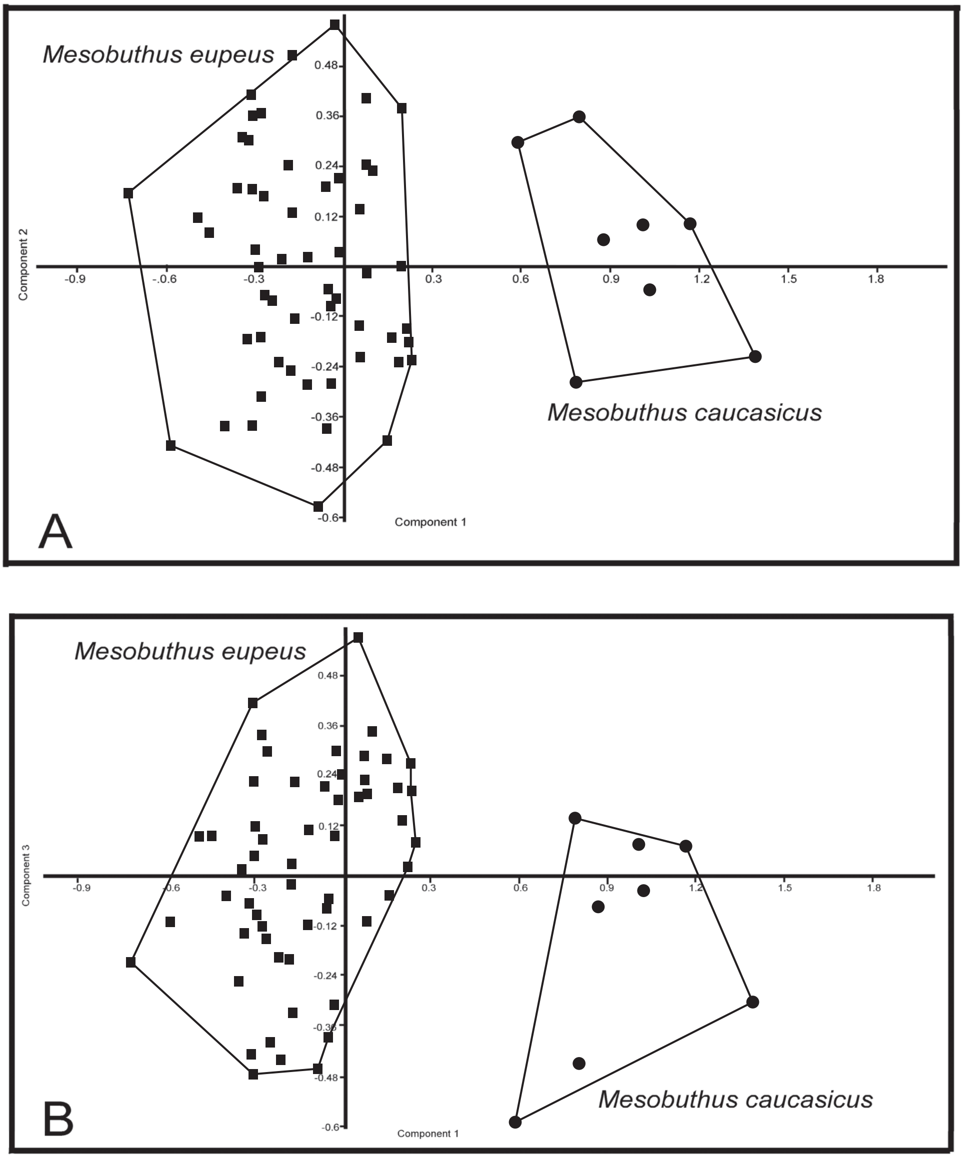

Figure 3. Principal component analysis of *Mesobuthus eupeus* and *Mesobuthus caucasicus* based on morphological ratios. (A) and (B) plot positions of the species, respectively, with respect to the first and second components and with respect to the first and third components (see text). *Mesobuthus eupeus* and *M. caucasicus* are encircled according to species identification in phylogenetic tree shown in Figure 2: ■, *M. eupeus*; ●, *M. caucasicus*.

total variation. Figure 3(A,B) clearly show that the first component was most effective in defining the divergence between the two species. Variables Mt5_l/h, Mt5_l/w and Tl_l/w had the highest positive loadings for this component, and two variables Ca_aw/pw and Ca_X/Y had negative loadings.

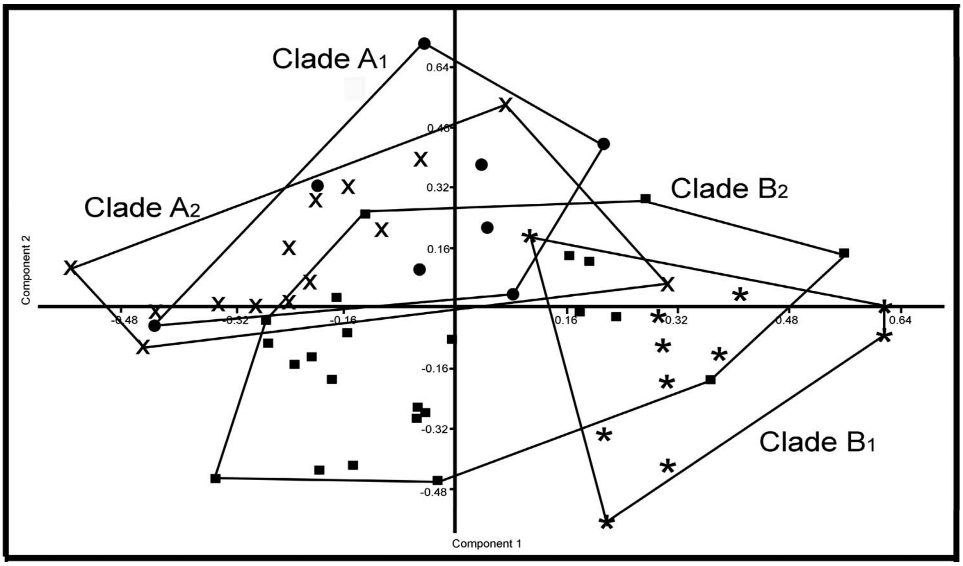

Figure 4. Principal component analysis of *Mesobuthus eupeus* based on morphological ratios. The specimens are plotted with respect to first and second components (see text). *Mesobuthus eupeus* specimens are encircled within clades according to phylogenetic tree shown in Figure 2: ●, *M. eupeus* clade A_1; **X**, *M. eupeus* clade A_2; ★, *M. eupeus* clade B_1; ■, *M. eupeus* clade B_2.

Principal component analysis based on the same 17 morphological ratios was also performed only for *M. eupeus* species (Figure 4). Notably, the analysis showed divergence between *M. eupeus* clade A and *M. eupeus* clade B. As expected, the divergence between these *M. eupeus* clades was notably less than between *M. eupeus* and *M. caucasicus* (Figure 3). For the *M. eupeus* species analysis, the first three components explained 28.83%, 26.02% and 15.09% of the total variation. Here, the second component was more effective in defining the divergence, and variables Ch_l/ml, Tl_l/w and Tl_l/h had positive loadings. Canonical discriminant analysis based on the 17 morphological ratios resulted in discrimination between *M. eupeus* clade A, *M. eupeus* clade B and *M. caucasicus* (Figure 5).

Discussion

A remarkable feature of the tree presented in Figure 2 is the poor resolution for certain clades. As stated by Rokas et al. (2002), the first reason for poor resolution is that the trees might represent an adaptive radiation, if the rate of speciation for a given time period is relatively high, interlineage differentiations are expected to be low. This would result in poorly resolved phylogeny, which, however, is an exact representation of the historical relationships between the taxa. Moreover, employing several loci which diverged at different rates and/or addition of more taxa, is probably an alternative way to obtain well-resolved phylogenetic trees. However, Gantenbein and Keightley (2004) used nine nuclear genes (3856 bp) in the study of the genus *Mesobuthus* in the eastern Mediterranean region, but the phylogenetic tree produced by their data set was also weakly supported. Therefore, despite the weak statistical

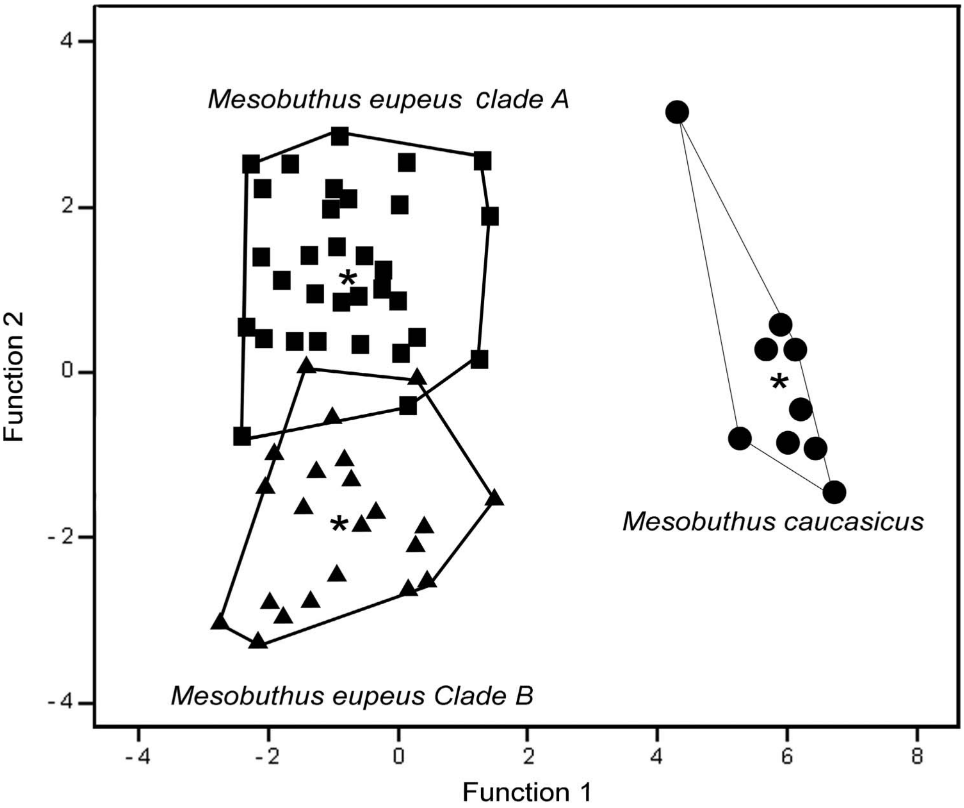

Figure 5. Canonical discriminant analysis of *Mesobuthus eupeus* and *Mesobuthus caucasicus* species based on morphological ratios. *Mesobuthus eupeus* and *M. caucasicus* are grouped according to species identification shown in Figure 2: ■, *M. eupeus* clade A; ▲, *M. eupeus* clade B; ●, *M. caucasicus*; ✱, group centroid.

support for major clades, the low nodal support of the resulting tree (Figure 2), is an accurate representation of the historical associations between the taxa analysed.

The *COI* sequence data presented here confirm the monophyly of *M. eupeus* as sister taxon of *M. caucasicus* (Figure 2). Furthermore, as expected, *M. gibbosus* and *M. cyprius* constituted a "Western clade" (89% bootstrap) well separated from the "Eastern clade" including *M. eupeus* and *M. caucasicus* (54% bootstrap). In the topology observed, the phylogenetic relationships within the Western clade were not clearly resolved as *M. gibbosus* was paraphyletic with respect to *M. cyprius*, suggesting that they may be synonymous. The latter species was described by Gantenbein et al.(2000) based on allozyme data and morphological evidence from Cyprus. Also, the data are consistent with *M. gibbosus* consisting of more than one species as proposed by Parmakelis et al. (2006). As the *M. gibbosus* and *M. cyprius* sequence data were retrieved from GenBank, we will not make a taxonomic decision on *M. cyprius* in this study.

With regards to *M. eupeus*, this taxon is a morphologically complicated species with 14 formally valid subspecies, of which five (Farzanpay 1987) to nine (Fet 1989; Fet and Lowe 2000) subspecies were described from Iran. It was considered that further phylogenetic analyses may result in elevation of some subspecies to the species level (Gantenbein et al. 2003). The *COI* data presented here confirmed the monophyly of *M. eupeus*. Additionally, all the analyses suggest subdivision of the species into distinct clades. Notably, the major subdivisions are supported by morphological comparisons and geographic distributions. They are also potentially explainable according to the geomorphological history of the Iranian Plateau. The most distinct division of *M. eupeus* shown by the resulting topology is between clade A and clade B. The phylogenetic tree is also compatible with further subdivision of clades A and B, respectively, into clades A_1 and A_2, and clades B_1 and B_2. Clade B_2 is potentially further divisible into $B_{2.1}$ and $B_{2.2}$ (Figure 2). The clade A_2 which was originally described as a separate species, *Buthus phillipsi*, was later downgraded to a subspecies of *M. eupeus* as *M. e. phillipsi* (Farzanpay 1987; Fet and Lowe 2000). Sequence divergence comparisons support these divisions, as the values for pairwise comparisons between *M. eupeus* clades (4–7%) approach those observed between distinct *Mesobuthus* species (5–11%) (Table 2). The highest divergence (7%) was observed between members of A_2 and $B_{2.2}$ clades.

Clade A members were all collected from the southern parts of Iran, whereas those of clade B were from a belt extending from southwestern Turkey (nominotypical subspecies *M. e. eupeus*) to the north (*M. e. philippovitschi*), northeastern Iran and southeastern Turkmenistan (*M. e. thersites*). The habitats of clade A are highly to moderately arid and distinctly different from the habitats of clade B. The habitats of clade B include humid environments on northern slopes of the Alborz Mountains, high altitude locations in northeastern and northwestern Iran and xeric habitats in the east of Iran. In general, specimens from the northern and northwestern parts of the range of distribution (Clade B) tend to be darker in colouration than specimens from further southern regions (Clade A). In the resultant topologies the nominotypical subspecies, *M. e. eupeus*, which is found in Turkey, Caucasus and northwest Iran, is highly supported, but the other northern sequences showed a more complicated case. Therefore, the taxonomic validity of the other northern subspecies, namely *M. e. philippovitschi* should be revised carefully based on the morphological characteristics and geographic data. Furthermore, the sequences from northeast and east of Iran could be assigning to the subspecies, *M. e. thersites* and *M. e. afghanus.* Finally, the relationship between southern sequences (Clade A) is concordant with two subspecies namely *M. e. phillipsi* (southwest) and *M. e. kirmanensis* (southeast).

Earlier morphological comparisons of *M. eupeus* had suggested existence of various subspecies (Birula 1900, 1905; Farzanpay 1987; Fet 1994). Reanalysis of morphological characteristics here by principal component and canonical discriminant analyses not only obviously distinguished between *M. eupeus* and *M. caucasicus*, but also supported the existence of *M. eupeus* clade A and *M. eupeus* clade B (Figures 3 and 4). The members of clade B are distinguished from those of clade A by having longer metasomal segments (I–III), relatively shorter fixed fingers and more inflated vesicles.

Topographic barriers that may have been responsible for distinction of the clades proposed here need to be considered. Our *COI*-based phylogenetic tree is compatible with clade A being ancestral to clade B (Figure 2). As distribution of clade A was distinctly within southern Iran and that of clade B was in northern Iran, a south to north distributional expansion in past history is implicated. This gradient may have been instigated by progressive expansion of aridization toward the north, making northern regions of Iran favourable habitats for scorpion species and promoting the migration northward. The proposed palaeoclimatic changes and aridization occurred during the Tertiary (Fet 1994; Gantenbein et al. 2003).

The present-day geographic isolation of clade A and clade B may have been affected by topographic barriers that evolved during or after the distributional expansion process. The relevant vicariant events here may have been the uplifting and formation of the Zagros and Alborz Mountain ranges, which occurred in the late Tertiary approximately 5–10 mya (Macey et al. 1998; Gök et al. 2003; Ramezani Oomali et al. 2008). The formation of Kavir and Lut deserts as a consequence of geomorphological events on the Iranian Plateau may act as barriers to dispersal. The effects of geomorphological features on the distribution of various African species of *Hottentotta*, *Parabuthus*, *Uroplectes* and *Opistophthalmus* have been previously reported (Lamoral 1979; Fet et al. 1998; Prendini 2005). Mountain systems have played the most important role in shaping the present distribution of various taxa, including the gekkonid lizards of the genus *Assacus* (Macey, et al. 1998; Rastegar Pouyani 2006). Rastegar Pouyani (2006) proposed that fragmentation resulting from uplifting of Zagros Mountains in the late Miocene or early Pliocene is a general scenario for the fauna of the Iranian Plateau.

Fossil evidences of *Mesobuthus* are lacking. However, time of separation of clade A and clade B can be estimated based on an assumed molecular clock. The time of divergence was estimated at 3.15–4.61 mya based on published divergence rates of *COI* (Quek et al. 2004). The 4.61-mya estimate may be more accurate because it is based on the relatively slower sequence divergence rate of 1.3% per million years. Variations in sequence divergence rates mainly depend on generation time and the rate of metabolism (Towler et al. 2001). Within arthropods, scorpions gave the longest generation time and they had the lowest known rate of metabolism among all animals (Martin and Palumbi 1993), suggesting that their sequence divergence rate should be relatively low. The estimated age of 4.61 mya (and even of 3.15 mya) for clade A and B corresponds to the early and middle Pliocene geological events of the Iranian Plateau and is consistent with the proposal that the formation of Zagros in the south and consequent uplifting of Alborz may have affected their evolution.

In conclusion, the phylogenetic analysis and sequence divergence data presented here based on *COI* sequence data indicate two distinct lineages within *M. eupeus* suggesting that it may be a species complex consisting of at least two species. Although, considerable morphological conservation is apparent for *M. eupeus*, statistical analysis of morphological features are consistent with the possible phylogenetic history being considered. Geographic distribution of *M. eupeus* and palaeogeographic evidences also support the proposal. However, as the molecular analysis was based only on the partial sequence of a single gene, a taxonomic decision about *M. eupeus* is not now warranted. We suggest further molecular testing using other genes and a detailed revision of diagnostic and morphological characteristics before a taxonomic decision is made.

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