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Genetic diversity within scorpions of the genus *Buthus* from the Iberian Peninsula: mitochondrial DNA sequence data indicate additional distinct cryptic lineages

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Abstract. Historically *Buthus occitanus* (Amoreux 1789) was recognized as the sole species of the genus present in the Iberian Peninsula, but recent morphological studies have identified and named two additional species. In addition, molecular data on the Moroccan fauna has shed light on the diversity within the genus. More species have since been described from North Africa, where diversity within the genus is highest. In this study we assessed the genetic diversity within specimens of *Buthus* Leach 1815 from across the Iberian Peninsula using cytochrome oxidase 1 (CO1) mitochondrial DNA sequences. The known range of *B. ibericus* Lourenço & Vachon 2004 was greatly expanded, with the species widespread in most of the western part of the Iberian Peninsula. Five distinct mtDNA lineages were found within *Buthus* from the Iberian Peninsula, two of which were reported for the first time in this study. However, both *B. ibericus* and *B. occitanus* included highly divergent lineages and thus further studies are needed to fully comprehend the taxonomy of *Buthus* from this region.

Keywords: CO1, phylogeny, Scorpiones

Historically only one species of the scorpion genus *Buthus* Leach 1815, *Buthus occitanus* (Amoreux 1789) was recognized from the Iberian Peninsula (Fet & Lowe 2000). Recently two new endemic species were described, *Buthus ibericus* Lourenço & Vachon 2004 and *Buthus montanus* Lourenço & Vachon 2004, from the southern Spanish provinces of Cadiz, and Granada and Almeria, respectively. These authors also suggest that *B. ibericus* may be present in the Algarve, southern Portugal, but these specimens were not included in their morphological analyses due to the poor state of the museum specimens examined. All other European specimens, from France and Spain, were attributed to *B. occitanus*. Later Teruel & Pérez-Bote (2005) examined a population of scorpions from Caceres Province in Extremadura (Central Spain) and concluded the specimens were also *B. ibericus*.

Phylogenetic and taxonomic assessments of *Buthus* in Iberia and North Africa have generally been in a state of rapid progression in recent years. As well as *B. ibericus* and *B. montanus*, several new species have been described from Morocco (e.g., Lourenço & Geniez 2005). Regarding their phylogenetic relationships, various studies have been performed using mitochondrial DNA (mtDNA) and nuclear DNA sequences (e.g., Gantenbein & Largiadèr 2003) and allozymes (Gantenbein 2004), and all have recognized that extensive cryptic genetic variation occurs. Gantenbein & Largiadèr (2003) found that European samples were highly distinct from North African populations, and could be divided into three distinct subclades. However, sampling in Europe was limited to 12 individuals from eight populations – four from the northeastern limit of the range in Catalonia (northeastern Spain) and France, and four from southern Spain and Portugal. Three of these southern populations, from Picacho and Benaocaz in Spain and Mértola in Portugal, could correspond to *B. ibericus*, which was described from this geographic region after this work, since they were genetically very distinct from the northern specimens of *B. occitanus*.

Despite this recent work, various basic aspects of species distribution, phylogeography and taxonomy of *Buthus* from the Iberian Peninsula remain unknown. Further, extensive sampling of this biodiverse region is needed to recover the complete phylogeographic pattern – genetically distinct lineages that appear to represent cryptic incipient species are still regularly being discovered in vertebrates (e.g., Pinho et al. 2006; Paulo et al. 2008). The aim of this study was therefore to assess genetic diversity within specimens from across the Iberian Peninsula using cytochrome oxidase 1 (CO1) mtDNA sequences, the gene typically included in barcoding studies (e.g., Hebert et al. 2003), and already employed by Gantenbein & Largiadèr (2003). These results are then compared to the specific status of the specimens to better assess diversity and distributions of the described forms.

METHODS

Information and geographic location of the specimens are given in Table 1 and Fig. 1. Adult and late instar specimens were examined morphologically, and identified to species level following Lourenço & Vachon (2004) and Teruel & Pérez-Bote (2005).

For the genetic analyses, whole genomic DNA was extracted from preserved (ethanol 96%) muscle tissue (leg fragment) using a standard high-salt protocol (Sambrook et al. 1989). A fragment of the cytochrome oxidase 1 (CO1) was amplified by polymerase chain reaction (PCR) using the primers from Folmer et al. (1994), LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′).

The PCR conditions (25 ml reactions) were as follows: each reaction contained 2.5 ml 10× Promega Buffer B, 0.5 ml 10 mM of each primer, 1.5 ml 25 mM MgCl2, 0.5 ml 10 mM dNTP's, 0.1 ml Promega Taq DNA polymerase and approxi-

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Table 1.—Localities of samples used, their position in Figure 1, and their respective MtDNA lineages in Figure 2; juvenile specimens could not be confidently identified to species levels and thus are represented as *Buthus* sp.; **a** - individuals identified before Lourenço & Vachon (2004) study; **α**, **β** and **γ** share haplotypes, only one of each is represented in Figure 2.

MtDNA lineage	*Buthus* Id	Taxonomy	Sex	Lat.	Long.	Country	GenBank Code
1	Sc084	*Buthus ibericus*	f	38.130	−7.019	Portugal	GQ168519
1	Sc089	*Buthus ibericus*	f	38.052	−7.028	Portugal	GQ168520
1	Sc095$^{\alpha}$	*Buthus* sp.	m	41.549	−6.231	Portugal	GQ168521
1	Sc100	*Buthus ibericus*	m	38.074	−7.046	Portugal	GQ168525
1	Sc104$^{\alpha}$	*Buthus* sp.	m	41.439	−6.324	Portugal	GQ168526
1	Sc105	*Buthus ibericus*	f	40.055	−7.193	Portugal	GQ168527
1	Sc106	*Buthus ibericus*	f	40.055	−7.193	Portugal	GQ168528
1	Sc107	*Buthus ibericus*	f	39.954	−7.119	Portugal	GQ168529
1	Sc108	*Buthus ibericus*	f	39.954	−7.119	Portugal	GQ168530
1	Sc109	*Buthus ibericus*	m	39.954	−7.119	Portugal	GQ168531
1	Sc112	*Buthus ibericus*	f	38.528	−8.004	Portugal	GQ168532
1	Sc113	*Buthus ibericus*	m	37.055	−8.924	Portugal	GQ168533
1	Sc114	*Buthus* sp.	m	37.022	−8.924	Portugal	GQ168534
1	Sc115	*Buthus ibericus*	f	38.163	−8.579	Portugal	GQ168535
1	Sc116	*Buthus ibericus*	m	38.685	−8.346	Portugal	GQ168536
1	Sc120	*Buthus ibericus*	m	38.528	−8.004	Portugal	GQ168537
1	Sc121	*Buthus ibericus*	f	37.186	−7.914	Portugal	GQ168538
1	Sc157	*Buthus ibericus*	f	39.433	−7.578	Portugal	GQ168539
1	Sc158	*Buthus ibericus*	f	39.512	−7.065	Spain	GQ168540
1	Sc161	*Buthus* sp.	f	39.360	−4.358	Spain	GQ168541
1	Sc190	*Buthus ibericus*	f	36.797	−6.378	Spain	GQ168542
1	Boo IB5a	*Buthus occitanus*[a]		37.717	−7.600	Portugal	AJ506911
1	Boo IB5b	*Buthus occitanus*[a]		37.717	−7.600	Portugal	AJ506912
2	Boo IB7a$^{\beta}$	*Buthus occitanus*[a]		36.534	−5.650	Spain	AJ517182
2	Boo IB7b$^{\beta}$	*Buthus occitanus*[a]		36.534	−5.650	Spain	AJ517183
2	Boo IB8	*Buthus occitanus*[a]		36.700	−5.417	Spain	AJ517184
3	Sc098	*Buthus* sp.	m	36.639	−5.248	Spain	GQ168523
3	Sc099	*Buthus occitanus*	m	36.639	−5.248	Spain	GQ168524
4	Sc096	*Buthus* sp.	f	37.740	−2.569	Spain	GQ168522
5	Boo IB1a$^{\gamma}$	*Buthus occitanus*[a]		43.488	3.558	France	AJ506905
5	Boo IB1b	*Buthus occitanus*[a]		43.488	3.558	France	AJ506906
5	Boo IB2	*Buthus occitanus*[a]		43.183	3.000	France	AJ506907
5	Boo IB3a$^{\gamma}$	*Buthus occitanus*[a]		42.433	3.117	France	AJ506908
5	Boo IB3b$^{\gamma}$	*Buthus occitanus*[a]		42.433	3.117	France	AJ506909
5	Boo IB4	*Buthus occitanus*[a]		42.050	2.582	Spain	AJ506910
5	Boo IB6$^{\gamma}$	*Buthus occitanus*[a]		36.831	−2.467	Spain	AJ517296
5	EU523755$^{\gamma}$	*Buthus occitanus*				Unknown	EU523755
	Bop MA1	*Buthus paris*		31.566	−7.686	Morocco	AJ506913
	Bop MA2	*Buthus paris*		31.738	−7.029	Morocco	AJ506914
	Bom HA3a	*Buthus mardochei*		30.918	−6.924	Morocco	AJ506896
	Bom HA3b	*Buthus mardochei*		30.918	−6.924	Morocco	AJ506897
	Bom HA6a	*Buthus mardochei*		30.943	−7.123	Morocco	AJ506901
	Bom HA6b	*Buthus mardochei*		30.943	−7.123	Morocco	AJ506902
	Bot TA1	*Buthus tunetanus*		32.523	8.054	Tunisia	AJ506916

mately 100 ng per ml DNA template. The cycle parameters were: initial denaturation at 94° C (3 min), denaturation at 94° C (30 s), annealing at 53° C (45 s) and extension at 72° C (45 s) repeated for 35 cycles. Amplified DNA templates were enzymatically purified and sequenced using the ABI PRISM BigDye Terminator protocols. The sequencing primers were the same as those used in the PCR reactions. Sequences were visualized on an ABI-310.

DNA sequences were aligned by eye, but this posed no problems as no indels were found. All new sequences were submitted to GenBank (Table 1). All available CO1 sequences from Iberian *Buthus* on GenBank were aligned to the new data, as were four samples of *B. mardochei* Simon 1878 from the High Atlas in Morocco that appear most closely related to Iberian specimens (Gantenbein & Largiadèr 2003). Three more distantly related samples of *B. tunetanus* (Herbst 1800) and *B. paris* (C.L. Koch 1839) (from Gantenbein & Largiadèr 2003) were included as outgroups following the estimated relationships presented by Gantenbein & Largiadèr (2003).

Sequences were imported into PAUP* 4.0b10 (Swofford 2003). For the phylogenetic analysis Maximum Likelihood (ML) and Maximum Parsimony (MP) approaches were used, with random sequence addition (100 replicate heuristic search with TBR branch swapping; in MP search all characters were equally weighted). Support for nodes was estimated with the bootstrap technique (Felsenstein 1985) using 1000 replicates for both ML and MP analyses. The AIC criteria carried out in Modeltest 3.06 (Posada & Crandall 1998) was used to choose

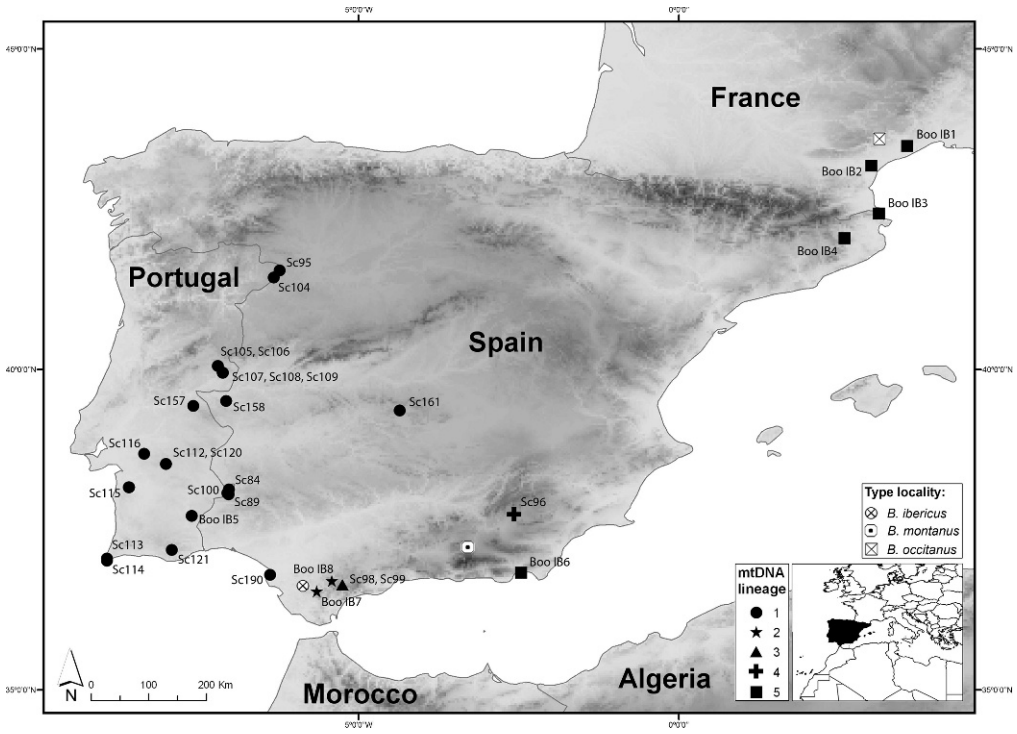

Figure 1.—Map showing the sampling locations of *Buthus* from the Iberian Peninsula and southern France included in this study. Samples are grouped in mtDNA lineages. Specimen codes follow Table 1. Type localities of the three *Buthus* species present in the Iberian Peninsula are also represented.

the model of evolution and the parameters for the ML analysis. Bayesian analysis was implemented using MrBayes v.3.1 (Huelsenbeck & Ronquist 2001), using a GTR + I + γ model with parameters estimated as part of the analysis and four incrementally heated Markov chains with the default heating values. The analysis was run for 10^6 generations, saving one tree in each 100 generations. The log-likelihood values of the sample point were plotted against the generation time, and all the trees prior to reaching stationary were discarded. The remaining trees were combined in a 50% majority consensus tree, in which the frequency of any particular clade represents the posterior probability (Huelsenbeck & Ronquist 2001).

All specimens are deposited in the collection of CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Vairão, Vila do Conde, Portugal.

RESULTS

In total, 24 new specimens were sequenced, from 19 locations in the Iberian Peninsula, for a total of 596 base pairs. Twenty-two new haplotypes were resolved, and combined with 15 from GenBank, from eight localities from Iberia and southwestern France, although these were slightly shorter fragments than the new sequences. Ninety polymorphic sites were found, 64 of which are parsimony informative. High levels of genetic variability were found in the analyzed sequences (Hd = 0.994, π = 0.05589, with similar levels within *Buthus occitanus* lineages: Hd = 1.000, π = 0.04723; and *B. ibericus* lineages: Hd = 0,990, π = 0.04098). Low proportion of G's (especially in 3rd position sites), lack of stop codons in the translated sequences, no insertions or deletions and similarity with published sequences all indicate these are mtDNA coding COI sequences, and not nuclear copies. Tajima's neutrality test was non significant (Tajima's $D = -0{,}47912$; $P > 0.10$).

The most appropriate model of evolution for this dataset was the general time reversible model, with an estimate of invariable sites (0.55) and of the gamma shape parameter (1.04). The ML analysis recovered a single tree (−ln 2588, Fig. 2). 1121 MP trees were recovered (337 steps), the strict consensus of which differed from the ML tree only at nodes with <50% support in either analysis (Fig. 2). The Bayesian analysis recovered a similar tree to the ML analysis, again differing only at weakly supported nodes.

DISCUSSION

As noted in *Buthus* (Gantenbein & Largiadèr 2003) and in the genus *Scorpio* Linnaeus 1758 (Froufe et al., 2008) mtDNA variation is very high in many phylogeographic studies of scorpions (15% and 10% nucleotide divergence respectively, compared to average between species of considerably less than this in vertebrates, Johns & Avise 1998). Here variation is again notable, with 23 of 25 new specimens sequenced having unique haplotypes. At least five highly differentiated lineages occur in the Iberian Peninsula. One clearly corresponds to *B. occitanus* and is found in the northeast part of the range (Boo IB1 and Boo IB2 from close to the type locality) and in a sample from Almeria (Boo IB6), in southern Spain. Variation within this clade is low (0.6%, K2P distance, Table 2), possibly indicating a rapid expansion, presumably from the south along the east coast of Spain towards the north. A separate clade includes all the samples from the western part of the

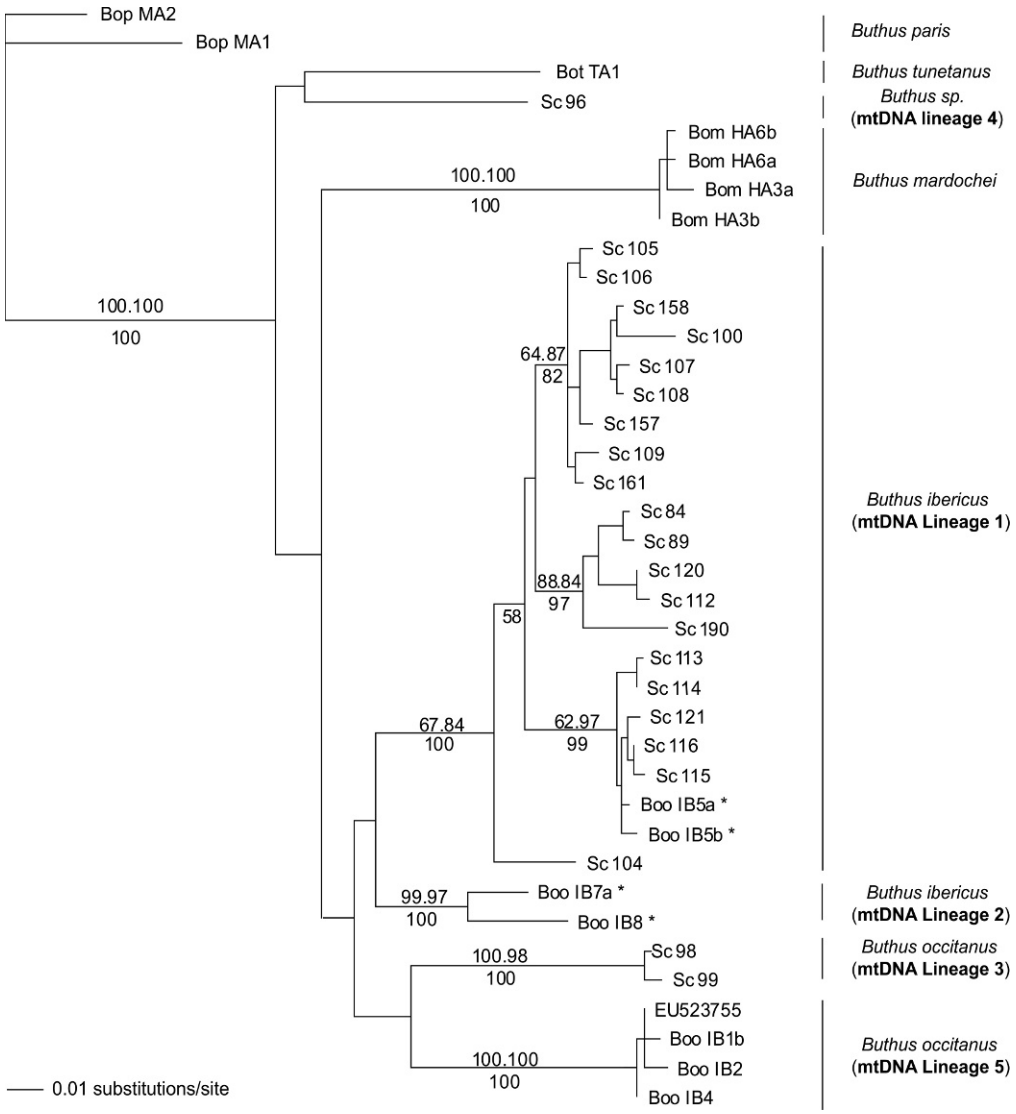

Figure 2.—Phylogram showing phylogenetic relationships estimated using maximum likelihood as described in the text. ML and MP bootstrap support is indicated above nodes, Bayesian posterior probabilities below nodes. The tree was rooted using three specimens of *B. tunetanus* and *B. paris*. Codes refer to Table 1. * indicate specimens identified as *B. occitanus* in Gantenbein & Largiadèr (2003), but assumed to correspond to the later-described *B. ibericus* based on their geographic origin and their position in the phylogenetic analysis.

Iberian Peninsula, including two samples from Mértola (Portugal) reported by Gantenbein & Largiadèr (2003). Substructuring within this clade seems to be present, with four subregions, although with relatively low support levels. Although the four subregions identified are generally geographically separate, in one area of Alentejo (southern Portugal) two sublineages occur in the same region (specimens Sc 84 and Sc 100). Three subregions occur in Central and Southern regions, but one (specimens Sc 95 and Sc 104) is currently only known from northern Portugal. However, extensive sampling would be needed to fully assess phylogeographic variation within this lineage.

Morphologically, all specimens that could be fully assessed from this clade appear to be *B. ibericus* following Teruel &

Table 2.—Net pairwise sequence divergence (Kimura 2-parameter) between the five lineages found in the Iberian Peninsula. In the last column values for within lineage divergence are present.

	lineage1	lineage2	lineage3	lineage4	within lineage estimates
lineage1	-				0.030
lineage2	0.043	-			0.043
lineage3	0.073	0.063	-		0.005
lineage4	0.085	0.062	0.074	-	-
lineage5	0.075	0.064	0.086	0.095	0.006

Pérez-Bote (2005). However, to complicate matters the samples reported from southern Spain by Gantenbein & Largiadèr (2003) are also from the area where *B. ibericus* was described (Figure 2, lineage 2), but form a highly distinct lineage (4.3%, K2P distance, Table 2). Adult *B. ibericus* are easily distinguished from *B. occitanus* by the presence of a node on the base of the movable finger of the chela (Lourenço & Vachon 2004). Unfortunately this is not clear in juveniles, making separation of the two species in early instars very difficult (Teruel & Pérez-Bote 2005). The previously reported presence of *B. occitanus* in western Iberia may be due to misidentification of specimens, or *B. ibericus* and *B. occitanus* may occur here in sympatry.

In this study two new and highly distinct lineages are also reported for the first time. One sample, Sc 96 (Figure 2, lineage 4), comes from the Sierra Nevada region and may correspond to *B. montanus*, but unfortunately the specimen is an early instar so could not be assigned with confidence to this species based on morphological characters. The other new lineage (samples Sc 98 and Sc 99; Figure 2, lineage 3), from southern Spain, morphologically resembles *B. occitanus*, but differs from the other specimens of this species by 8.6% (K2P distance from lineage 5, Table 2).

Although all five lineages are highly distinct (Table 2), relationships between them are poorly supported (Fig. 2). In MP no relationship between lineages has >50% bootstrap support. In the ML analyses the Iberian forms are not monophyletic, with one specimen (Sc 96) being the sister taxon to a specimen from Tunisia, although again support levels between lineages are low. In the Bayesian analysis the Iberian forms are monophyletic (posterior probability 23), with the specimen Sc 96 sister taxon to the *B. occitanus* mtDNA lineage 3 (posterior probability 41; Fig. 2). These differences have very low support levels and highlight only that relationships between lineages remain essentially unresolved.

The Iberian Peninsula, along with other southern European regions such as Italy and the Balkans, has long been viewed as an important refugia for biodiversity that was greatly reduced in northern Europe during Pleistocene glaciations (Hewitt 2000). Recent phylogeographic studies have extended this line of reasoning stressing the cryptic diversity found in the Iberian Peninsula, particularly in the southeast as evidence for "refugia within refugia" (reviewed in Gómez & Lunt 2007). This seems likely to be due to the complex geology of the region, especially during the Miocene when, prior to the Messinian salinity crisis, much of this region was an archipelago, allowing allopatric differentiation and also linking North African and European forms (reviewed in Paulo et al. 2008). *Buthus* is another example of this cryptic diversity, with multiple distinct lineages in the region, some of which appear to be more similar to North African lineages than to other Iberian lineages.

Although genetic diversity within *Buthus* from the Iberian Peninsula is very high, it is widely accepted as essentially impossible to consistently delimit species based solely on pairwise distances (Meier et al. 2006). In their study of 449 species of Diptera, Meier et al. (2006) showed that 33% of currently accepted species harbored lineages with greater than 3% CO1 sequence divergence, and thus would be considered species complexes if this was used as a threshold value. On the other hand, various recent barcoding studies have identified cryptic species based on CO1 divergences less than this (e.g. Ståhls et al. 2009). The divergence between lineages reported in this study also vary considerably from 4.3 to 9.5%, further highlighting the difficulties in defining thresholds based on single markers. Therefore the finding of these lineages within *Buthus* does not on its own indicate that undescribed species exist in the Iberian Peninsula, but the highly divergent lineages do warrant further investigation.

In conclusion, at least five distinct mtDNA lineages occur within *Buthus* from the Iberian Peninsula, two of which are reported for the first time in this study. Relationships between lineages are not well supported, but Iberian forms do not appear to be monophyletic. Specimens morphologically attributable to the recently described *B. ibericus* are widespread in the western part of the Iberian Peninsula, greatly expanding the range of this species. Additional fieldwork and analysis of museum specimens is necessary to clarify ranges of the newly described Iberian species. Of particular importance is the collection of specimens from the type locality of *B. ibericus*, in order to clarify the identity of one of the mtDNA lineages (lineage 2) found in this study. However, both *B. ibericus* and *B. occitanus* include highly divergent lineages, and thus more detailed morphological analyses, together with the analysis of nuclear genes, are needed to redefine the taxonomy of *Buthus* from this region.

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