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Genetic diversity of Maghrebian *Hottentotta* (Scorpiones: Buthidae) scorpions based on CO1: new insights on the genus phylogeny and distribution

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ABSTRACT

The medically important scorpion genus *Hottentotta* Birula, 1908 has long been a taxonomical challenge. This species-rich scorpion genus contains three lineages spread over most of Africa and part of Asia. The Maghrebian *Hottentotta* was historically recognised as a single species, *H. franzwerneri* (Birula, 1914), divided in two subspecies with disjunct distributions. A recent morphological study raised both Maghreb subspecies to species level, *H. franzwerneri* and *H. gentili* (Pallary, 1924). In this study we assess the phylogenetic relationships between specimens of the genus *Hottentotta* from Morocco using cytochrome oxidase 1 (CO1) mitochondrial DNA sequences. Our finding of *H. gentili* in the eastern portion of Morocco increases the known range of this taxon and significantly reduces the geographic distance that separates it from *H. franzwerneri*. Furthermore, we found four well supported clades in the Maghrebian *Hottentotta*. All *H. franzwerneri* specimens group in the *franzwerneri* clade, but *H. gentili* specimens group in three different clades. The Ziz valley clade form a sister group to the *franzwerneri* clade, specimens from the core range of *H. gentili* group in the central clade, while specimens from the southern distribution of the species group in the Low Draa valley clade, basal in our tree. These findings challenge current *Hottentotta* taxonomy because they imply paraphyly of *H. gentili*, although mitochondrial introgression cannot be excluded. Further studies are needed to fully comprehend the taxonomy of *Hottentotta* from this region and the role that colour characters play in scorpion species diagnoses.

KEY WORDS: Scorpiones, *Hottentotta*, Maghreb, mitochondrial DNA, CO1, phylogeny, taxonomy, colour, cryptic diversity.

INTRODUCTION

The scorpion genus *Hottentotta* Birula, 1908 is a widespread and diverse genus. Placed in the Buthidae C.L. Koch, 1837, the largest scorpion family, it comprises about 35 species that are found across Africa, the Arabian Peninsula and in Asia as far east as India (Kovařík 2007). The position of the genus *Hottentotta* relative to other buthids has not been firmly resolved. Taxonomic relations with the genus *Mesobuthus* Vachon, 1950 remain uncertain based on morphological data (Fet & Lowe 2000). To date the only study that tried to resolve the phylogeny of the Buthidae using DNA sequence data placed *Hottentotta* as the sister taxon to *Buthacus* Birula, 1908 (Fet *et al.* 2003). It should be noted, however, that the latter study only employed a short fragment of the rapidly evolving 16S rRNA gene to resolve the relatively deep splits in the family Buthidae.

The species diversity within the *Hottentotta* genus has been grouped in three lineages: the African, the Saharo-Sindian and the Indian. These lineages have been proposed based on morphological data alone (Birula 1914) and their relationships remain largely unresolved. The Maghreb representatives of this genus are placed in the Saharo-Sindian lineage, whose closest relatives can be found only in Egypt. The Maghreb *Hottentotta* have long been classified as a single species, *Hottentotta franzwerneri* (Birula, 1914) with two accepted subspecies with disjunct distributions: *H. f. franzwerneri* (Birula, 1914) and *H. f. gentili* (Pallary, 1924) (Fet & Lowe 2000). In 2007, Kovařík produced the most comprehensive revision to date of the genus *Hottentotta*. In this work the author elevated *H. gentili* (Pallary, 1924) to species status, stressing that the differences found in leg coloration, yellow in *H. franzwerneri* and black in *H. gentili,* were enough to make such a taxonomic change. Besides this clear morphological difference, the only other difference found between the two species was the presence of slight sexual dimorphism in the metasoma of *H. franzwerneri*, not observed in *H. gentili*. In his review of the genus, Kovařík (2007) also used colour characters to separate other groups of species.

Little is known about both species' ecology, although it is clear that *H. gentili* has a much wider distribution, approximately three times that of *H. franzwerneri*. As a result *H. gentili* can be found over a much larger altitudinal range, and thus in different climatic conditions, ranging from the partially snow-covered mountains of the High and Anti Atlas down to the Saharan plains. In comparison, *H. franzwerneri* is found on the lower Ksour Mountains of the Saharan Atlas Range and in the south-projecting plateaux, areas dominated by a Saharan climate. Both species, even if occurring in dry areas, are associated with more humid micro-habitat conditions (Vachon 1952). This ecological requirement brings then into close contact with human settlements. Disregarded until recently as a potential threat, *H. gentili* was found as an important cause of scorpion envenomation in the Moroccan southwest, being responsible for several deaths in the region (Touloun *et al.* 2001). To our knowledge, no data regarding the specific toxicity or composition of *H. franzwerneri* venom have been published. Given their medical importance, understanding the distribution of the genus's diversity in the region is important, because the correct identification of scorpion species is essential to the treatment of envenomation (e.g., Touloun *et al.* 2001).

The Maghreb region is highly biogeographically diverse, and cryptic diversity has recently been uncovered in both the Maghreb vertebrates (e.g., Lima *et al.* 2009) and the scorpion fauna (Gantenbein & Largiadèr 2003). The aim of this study is therefore to assess genetic diversity of *Hottentotta* specimens from Morocco using cytochrome oxidase 1 (CO1) mtDNA sequences, the gene used in barcoding studies (e.g., Hebert *et al.* 2003). Our sequence data show a strikingly different picture of the Maghrebian *Hottentotta* taxa to that found using morphological data alone.

MATERIAL AND METHODS

Information and geographic location of the specimens, all captured in Morocco, are given in Table 1 and Fig. 1. All specimens were examined morphologically, and identified to species level following Vachon (1952) and Kovařík (2007). All specimens are deposited in the collection of CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, Vairão, Vila do Conde, Portugal.

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

The PCR conditions (25 µl reactions) were as follows: each reaction contained 2.5 µl 10× Invitrogen PCR Buffer, 0.5 µl 10 mM of each primer, 1.5 µl 50 mM $MgCl_2$, 0.5 µl 10 mM dNTP's, 0.1 µl Invitrogen Taq DNA Polymerase and approximately 100 ng per µl DNA template. The cycle parameters were: initial denaturation at 94 °C for 3 min, denaturation at

TABLE 1

Localities of samples used, their position in Fig. 1, their respective Clade in Fig. 2, and corresponding GenBank accession numbers. Coordinates are in the WGS84 datum, in decimal degrees.

Clade	Field number	Taxon	Location	Lat.	Long.	Country	GenBank accession number
franz-werneri	Sc842	*H. franzwerneri*	Figuig outskirts	32.087	-1.241	Morocco	JF820094
franz-werneri	Sc864	*H. franzwerneri*					JF820095
Central	Sc041	*H. gentili*	3 km SSE of Tazidra, on road N8	30.990	-9.040	Morocco	JF820075
Central	Sc139	*H. gentili*	3 km E of Tirhmi, on road R104	29.580	-9.396	Morocco	JF820077
Central	Sc154	*H. gentili*	14 km NE of Assa, on road P1801	28.686	-9.319	Morocco	JF820081
Central	Sc173	*H. gentili*	Oued Draa valley, on N9, 8 km ESE of Agdz	30.668	-6.380	Morocco	JF820082
Central	Sc429	*H. gentili*	5 km WSW of Adrar Ounas	30.,895	-8.805	Morocco	JF820083
Central	Sc433	*H. gentili*	Oued Assaka valley, 25 km SE of Sidi el Hosaín	29.068	-10.248	Morocco	JF820084
Central	Sc534	*H. gentili*					JF820089
Central	Sc434	*H. gentili*	Oued Draa valley, on road N9, 6 km N of Agdz	30.746	-6.449	Morocco	JF820085
Central	Sc435	*H. gentili*					JF820086
Central	Sc449	*H. gentili*	2.5 km WNW of Jbel Habou el Khal	32.133	-3.155	Morocco	JF820087
Central	Sc795	*H. gentili*	Jebel Bou Arfa, 6 km NW of Bouârfa	32.571	-2.015	Morocco	JF820090
Central	Sc802	*H. gentili*	On road N10 to Bouanane, 19 km NE of the town	32.114	-2.884	Morocco	JF820091
Central	Sc803	*H. gentili*					JF820092
Ziz valley	Sc452	*H.gentili*	Oued Ziz valley, on road N13, 3 km SE of Oulad Aissa	31.744	-4.198	Morocco	JF820088
Ziz valley	Sc804	*H. gentili*					JF820093
Low Draa valley	Sc137	*H. gentili*	6 km ESE of Elkha-lona, on R101 heading S from Tan-Tan	28.028	-11.357	Morocco	JF820076
Low Draa valley	Sc142	*H. gentili*	On right margin of Oued Draa valley, 2 km W of the intersection with road N1 heading N from Tan-Tan	28.544	-10.957	Morocco	JF820078
Low Draa valley	Sc143	*H. gentili*					JF820079
Low Draa valley	Sc144	*H. gentili*					JF820080
	Sc292	*Androctonus mauritanicus*	1 km NE of Mechra Benabbou, on road N9	32.661	-7.793	Morocco	JF820097
	–	*Androctonus australis*	–	–	–	–	AF370829
	Sc002	*Buthus* sp.	On road N13, 4 km N from Ain Defali	34.630	-5.538	Morocco	JF820096
	–	*Centruroides vittatus*	–	–	–	USA	EU381060
	–	*Mesobuthus eupeus*	–	–	–	Iran	HM567390
	Sc051	*Scorpio fuliginosus*	–	–	–	Morocco	FJ198060
	–	*Tityus nematochirus*	–	–	–	Venezuela	FJ525423
	–	*Zabius fuscus*	–	–	–	Argentina	FJ525421

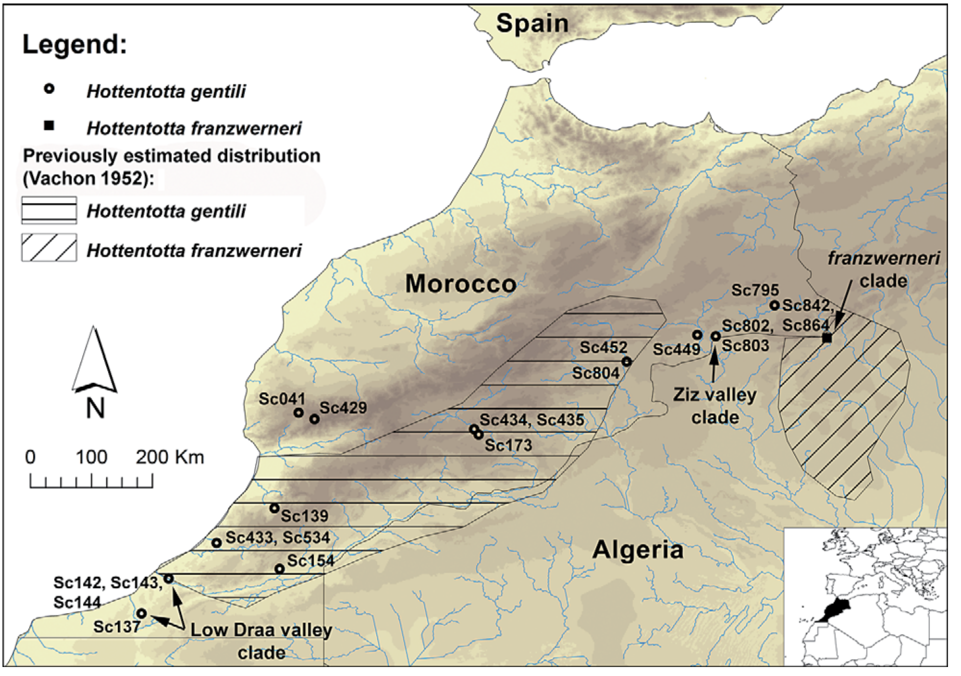

Fig. 1. Map showing the sampling locations of *Hottentotta* from Morocco included in this study. The estimated distribution of both *Hottentotta* species proposed by Vachon (1952) is indicated. The clades resolved in Fig. 2 are noted. Specimen codes follow Table 1.

94 °C (30 s), annealing at 52 °C (45 s) and extension at 72 °C (45 s) repeated for 35 cycles and a final extension at 72 °C for 5 min. 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 PCRs. Sequences were read on an ABI-310.

Sequences of seven Buthidae taxa, *Androctonus australis* (L., 1758), *A. mauritanicus* (Pocock, 1902), *Buthus* sp., *Centruroides vittatus* (Say, 1821), *Mesobuthus eupeus* (C.L. Koch, 1839), *Tityus nematochirus* Mello-Leitão, 1940, *Zabius fuscus* (Thorell, 1876) and one Scorpionidae taxon: *Scorpio fuscus* (Ehrenberg, 1829), were used as hierarchical out-groups.

Chromatograms were checked by eye using ChromasPro 1.41 (technelysium.com.au) and the sequences were subsequently aligned using ClustalW as implemented in MEGA 4 (Tamura *et al.* 2007) using the default settings. The resulting alignment was checked by eye, but was not found to require additional editing. Phylogeny reconstruction was performed using Maximum Likelihood (ML) and Bayesian Inference (BI) methods. The best fitting models of sequence evolution were determined by the AIC criterion in Modeltest 3.7 (Posada & Crandall 1998). ML tree searches were performed using PhyML, version 2.4.4 (Guindon & Gascuel 2003). Bootstrap branch support values were calculated with 1000 replicates. The BI analysis was conducted with MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001), using models estimated with Modeltest under the AIC criterion, with 5,000,000 generations, sampling trees every 10th generation (and calculating a consensus tree after omitting the first 12,500 trees). Log likelihood scores for the remaining trees were examined in Tracer 1.4 (http://beast.bio.ed.ac.uk/Tracer) and the appropriateness of the burnin-period was checked. Genetic variability was calculated with DnaSP v.5.10.01 (Librado & Rozas 2009), excluding sequences Sc434 and Sc435 due to a section of missing data (close to 200 bp) in both sequences.

In order to calculate the average genetic distances found between species recognized in genera of related Buthidae scorpions, CO1 sequences of *Centruroides* Marx, 1890 and *Mesobuthus* Vachon, 1950 were downloaded from GenBank and aligned, resulting in alignments of 39 and 19 sequences respectively. Genetic distances were calculated using MEGA 4 with Jukes-Cantor correction, using pairwise deletion of gaps and missing data, with several sequences per species when available. The alignments used are available from the authors upon request.

RESULTS

The alignment used in the phylogeny reconstruction consisted of 21 new DNA sequences from *Hottentotta* specimens collected in 14 locations covering most of southern Morocco. Additionally, seven outgroup sequences were used in the analysis (see Table 1). From the sequences produced, 16 haplotypes were resolved. The alignment had a length of 639 base pairs, with 92 polymorphic sites of which 87 were parsimony informative. High levels of genetic variability were found in the analysed *Hottentotta* sequences (Hd = 0.98, $\pi = 0.065$).

The recovered ML and BI trees did not differ in their topologies in any branch with moderate to high support (Bayesian posterior probability of over 0.83, see Fig. 2).

Four highly supported clades were retrieved within *Hottentotta*. Thirteen specimens from the core range of *H. gentili* grouped together in a single Central clade that grouped with little internal support. The clade consisting of two *H. franzwerneri* specimens nested strongly within *H. gentili* clades (Bayesian posterior probability of 1; Fig. 2). The sister group of the *H. franzwerneri* clade consists of two specimens collected in the Oued Ziz valley. Interestingly, these two specimens were not the closest geographically to the *franzwerneri* clade, this was specimen Sc795 (Fig. 1). Noticeably, the four specimens from southern Morocco grouped together in a basal clade in relation to the remaining *Hottentotta* specimens.

Our sampling effort significantly increased the known distribution of *H. gentili* to the eastern portion of Morocco.

DISCUSSION

Our study of *Hottentotta* scorpions found high levels of genetic diversity, retrieving 16 haplotypes in 21 specimens analysed, a result also reported by previous studies conducted on scorpions of the Maghreb and Iberian Peninsula, such as *Buthus* Leach, 1815 (Gantenbein & Largiadèr 2003; Sousa *et al.* 2010) and *Scorpio* L., 1758 (Froufe *et al.* 2008). More unexpected was the subdivision of two species into four well supported clades. More than half of all *H. gentili* specimens analysed grouped together in a clade containing specimens collected in the centre of the species' known range. Also noteworthy is the grouping of our *H. franzwerneri* specimens well within *H. gentili* clades (above 94 % bootstrap support). The inclusion of *H. franzwerneri* in the *H. gentili* clade may be explained by two different hypotheses. If *H. gentili* is a monophyletic species, then *H. gentili* mitochondrial introgression may have occurred, leaving a mark on the mitochondrial DNA of the *H. franzwerneri* specimens. On the other hand, if mitochondrial introgression has not confounded the resolution of the actual relationships of the clades of the Maghreb *Hottentotta*, the current taxonomy would need revision since this finding suggests that *H. gentili* as currently recognized may be a paraphyletic species. The existence of cryptic species that can only be uncovered using

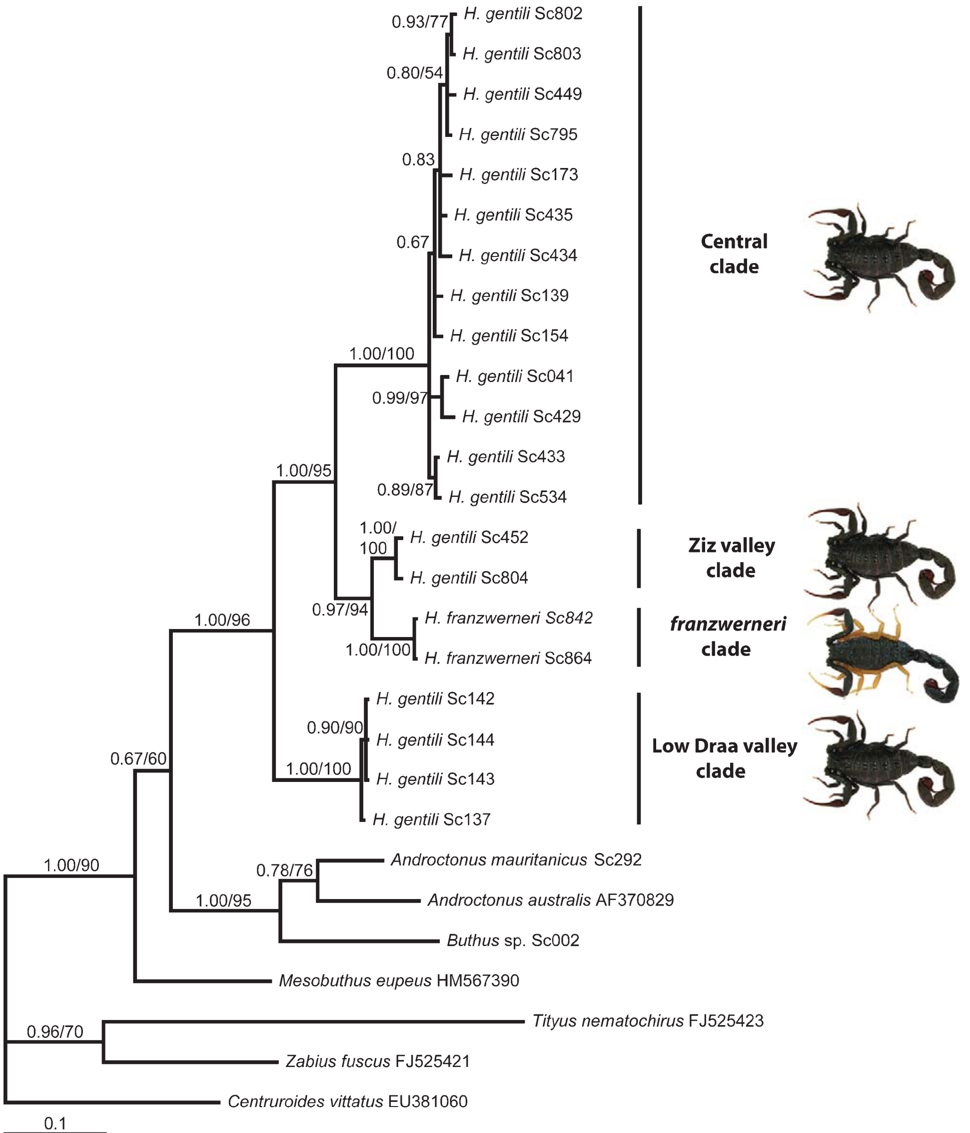

Fig. 2. Phylogram showing phylogenetic relationships estimated using Bayesian Inference as described in the text. Numbers at branches are Bayesian posterior probabilities and ML bootstrap percentages respectively. The tree was rooted with *Scorpio fuliginosus* (not shown). Codes refer to Table 1.

molecular characters seems to be a common pattern in scorpions (e.g., Gantenbein *et al.* 2000), due to a paucity of informative morphological characters in many taxa. This may lead to an over-evaluation of single morphological characters in delimiting species. In this case the use of colour alone to separate species within the *Hottentotta* genus must be re-evaluated in light of this new finding, as this was the only distinctive character used by Vachon (1952) and Kovařík (2007) to separate these taxa. Kovařík (2007) established *H. gentili* and *H. franzwerneri* as distinct species, but mentioned only the leg coloration and slight differences in sexual dimorphism of the metasoma and chela in *H. franzwerneri*. The latter differences were not found by Vachon (1952) although this author studied a similar number of adult specimens of both sexes of *H. franzwerneri* compared to Kovařík (2007). Both Vachon and Kovařík considered these species also geographically disjunct, with a minimum distance of around 200 km between their areas of distribution (Fig. 1). Nevertheless, the discover of *H. gentili* in the proximity of Bou Arfa (specimen Sc795) reduces the known distance between both species to around 70 km, and, more importantly, strongly suggests either that both species can be in contact in the present or that they have been in contact as recently as around 6,000 years ago, in the last wet phase in North Africa (deMenocal *et al.* 2000; Kuper & Kröpelin 2006).

Ecologically *H. gentili* and, to a lesser extent, *H. franzwerneri* are found in a wide variety of habitats and altitudinal gradients, although as suggested by Vachon (1952) the Maghreb *Hottentotta* are not true desert species. Even if they can be found in the south of Morocco, they appear to exist only in those places that can provide enough soil humidity, which in the drier south can be restricted to oases and river valleys. This factor may explain the connectivity found between *H. franzwerneri* and the Ziz valley clade if we assume that rivers provide corridors for dispersal.

The finding of a clade in the Low Draa Valley was also unexpected. This basal clade is the most genetically divergent according to our CO1 data, and must have separated early from the main Maghreb *Hottentotta* clade. We hypothesize that a continuously flowing Draa River, rather than seasonally flowing as is currently the case (abrupt changes in North Africa river basins are documented, e.g., Osborne *et al.* 2008), may have formed a biogeographic barrier. Other scorpion species only known from the south of the Draa River drainage in Morocco, including *Buthus bonito* Lourenço & Geniez, 2005 and *Microbuthus maroccanus* Lourenço, 2002, show that the Draa River may act as a barrier for scorpions. *Buthus rochati* Lourenço, 2003 can also be included in this pattern, because this species is only known from a region adjacent to the north of the drainage basin. The locality of specimen Sc137 suggests that the distribution of the species may extend further south than was reported by Vachon (1952) and Kovařík (2007), as can be seen in Fig. 1.

However it is noteworthy that the closest relatives of the Maghreb *Hottentotta* can only be found in Egypt [*H. minax* (L. Koch, 1875), Saharo-Sindian lineage] or south of the Sahara desert [e.g. *H. hottentotta* (Fabricius, 1787), African lineage] (Vachon & Stockmann 1968). This distribution pattern is remarkably different from other scorpions that show similar habitat preferences. In comparison, *Buthus* species can be found across North Africa except for the true desert areas (Vachon 1952). This is a further indication that the Maghreb *Hottentotta* require higher humidity in microhabitat conditions when compared, for example, with *Buthus* species.

In order to compare the genetic distances we found between the different clades of *Hottentotta*, we calculated the Jukes-Cantor corrected genetic distance between species of two different buthid genera. Based on 19 *Centruroides* species for which CO1 sequences were available in GenBank, we found an average genetic distance between species of 11.2 %, with a standard deviation of 2.6 %. A similar analysis was made on CO1 sequence data available for five species of *Mesobuthus*, which showed an average genetic distance between species of 15 %, with a standard deviation of 2.4 %. These are similar to the distances found in our study (12.1 %; Table 2) between the lower Draa clade and the Central clade, further suggesting that this clade may merit species status.

TABLE 2

Net pairwise sequence divergence (Jukes-Cantor) between the four clades found in Maghreb *Hottentotta*. Within brackets is the value for within lineage divergence for the Central clade.

	Central	Low Draa valley	Ziz valley
Central	(0.017)		
Low Draa valley	0.121	–	
Ziz valley	0.086	0.113	–
franzwerneri	0.098	0.116	0.049

In conclusion, four well-supported clades were found in the two species of *Hottentotta* from the Maghreb. These suggest the paraphyletic positioning of *H. franzwerneri*, although as our data derive from mtDNA alone, an ancient mitochondrial introgression event from *H. gentili* cannot be excluded. The existence of a putative cryptic species in the south of Morocco, possibly related with the lower Draa River is proposed. Additional fieldwork in the South of Morocco and adjacent areas of Algeria (a current conflict zone due to border issues between both countries), together with the analysis of nuclear genes, are necessary to clarify the taxonomic identity of *H. franzwerneri* and the existence of a cryptic species in the southern area of the Draa River.

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