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Intraspecific molecular variation among *Androctonus crassicauda* (Olivier, 1807) populations collected from different regions in saudi arabia

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ABSTRACT

Androctonus crassicauda is one of the medically important scorpion species occurring in the Middle East. In this study, molecular variation in mtDNA cytochrome oxidase subunit I (COI) gene within seven populations of *A. crassicauda* from four main ecological regions of Saudi Arabia was investigated. Scorpion specimens were collected from different eco-geographical regions. DNA was extracted, subsequently 700 bp fragments of COI gene were amplified using specific scorpion primers and sequenced. The obtained sequences were analyzed, and phylogenetic trees were constructed. The phylogenetic analysis showed high levels of genetic variation among *A. crassicauda* populations with the existence of three distinct lineages. Also, it supports the existence of two distinct populations of *A. crassicauda* in Saudi Arabia, which perhaps suggestive of a putative distinct species. Further morphological studies with additional specimens from the Arabian Peninsula may reveal possible undiscovered and cryptic species in the region.

1. Introduction

Androctonus Ehrenberg, 1828 is a genus of the Family Buthidae, with 30 described species (Rein, 2021; Yağmur, 2021). This genus was introduced by Ehrenberg in Ehrenberg and Hemprich (1828) with two sub-genera *Prionurus* Ehrenberg (=*Buthus*) and *Leiurus* Ehrenberg. Several studies dealt with species of this genus since Vachon (1948, 1952) standardized a definition of the genus *Androctonus*, contributing significantly to our growing knowledge on the systematics of this genus (Fet et al., 2000; Lourenço, 2005, 2008; Lourenço and Qi, 2006, 2007; Teruel et al., 2013; Kovařík and Ahmed, 2013; Lourenço et al., 2009, 2012, 2015; Rossi, 2015). Of these, *Androctonus crassicauda* (Olivier, 1807) is the most widely distributed species and known for its medical importance (Chippaux and Goyffon, 2008; Alqahtani and Badry 2021). The distribution of this species extended from Sinai, Egypt across Arabia, and the Middle East (Crucitti, 1999; Amr et al., 2021). It s venom contains toxins of which block potassium channels (Miller 1995), chloride channels (DeBin et al. 1993) and toxins which increase the flow of sodium ions into neurons to increase the excitability

Abbreviations: COI, cytochrome oxidase subunit I; mtDNA, mitochondrial DNA; MP, Maximum-parsimony; NJ, neighbor-joining; BI, Bayesian inference.

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of neurons (Zlotkin et al. 1994; Benkhalifa et al. 1997). Given its medical importance and widespread distribution understanding the distribution of the species diversity in the region is important, because the correct identification of scorpion species is essential to the treatment of envenimation. Recently, molecular phylogeny of scorpions is considered very useful to provide evidence of genetic heterogeneity within and between populations of different taxa (Gantenbein et al. 1999; Ben Ali et al. 2000; Fet et al. 2003; Gantenbein and Largiadèr 2003; Ben Othmen et al. 2009; Ozkan et al., 2010). We, therefore, employed molecular analysis to assess the presence of intraspecific molecular variations within *A. crassicauda* populations from four main ecological regions of Saudi Arabia, by sequencing the intraspecific hypervariable region of the mtDNA cytochrome oxidase subunit I (COI) gene.

2. Materials and methods

Biological material. Specimens of *A. crassicauda* were collected from nine location representing four main ecological regions in Saudi Arabia, including the North Arabian Desert (NAD), Central Arabian Desert (CAD), the Arabian Sand Desert (ASD), Southwestern Arabian Escarpment and Highlands (SAEH) (Fig. 1, Table1). Scorpions were collected during the period from January 2021 to July 2021, using mainly ultraviolet lights at night and randomly searched in their hiding places during the daytime (Williams 1968; Stahnke 1972). The collected scorpions were preserved in 95%ethanol for DNA isolation as described by (Prendini et al., 2003).

DNA extraction, COI-PCR amplification, and sequencing. Genomic DNA was extracted from fresh or preserved (in 95% ethanol) scorpions either from the pedipalps or from the muscle tissue of the legs using a DNeasy extraction kit (Qiagen). The 5́ hypervariable region of the mtDNA cytochrome oxidase subunit I (COI) gene was amplified by polymerase chain reaction (PCR) in all samples, using the universal COI primers according to Folmer et al. (1994) and Coelho et al. (2014). Products of the PCR were electrophoresed in a 1 % agarose gel stained with 0.5 μg mL^{-1} of ethidium bromide, observed by using UV transilluminator, purified, and then sequenced on an ABI 3500 automated sequencer (Applied Biosystems Inc., USA).

Phylogenetic analysis. Sequences were screened and analyzed using Finch TV 1.4.0 (Geospiza, Inc., USA; https://www. geospiza.-com). Additional sequence data were downloaded as ingroup from GenBank for *A. crassicauda* from Iraq and Iran (Table 1). Additional sequences data of COI region of congruent *Androctonus* species were also obtained from the Genbank and used as in-groups, including *A. amoreuxi*, *A. australis*, *A. bicolor*, *A. gonneti*, *A. liouvillei* and *A. mauritanicus* (Accession Numbers: KJ538436.1, KJ538184.1, KJ538333.1, KJ538381.1, KJ538220.1, and JF820097.1) respectively. Sequences data of *Scorpio palmatus* was downloaded as an outgroup (Accession Numbers AY156585.1). Obtained sequences were aligned with the ClustalW extension in MEGA 6 (Kumar et al. 1994), using the default settings. Nucleotide composition was calculated from the in-group sequences only. MEGA 6 was also used to estimate the genetic distances (for the entire data set). For the phylogenetic analysis, the mitochondrial COI gene data set (n = 38) was examined. The phylogenetic analysis (Maximum-parsimony (MP), Neighbor joining (NJ), and Bayesian inference (BI) were performed as described by Alqahtani and Badry (2020a). The Maximum-parsimony (MP) and neighbor-joining (NJ) analyses were performed with Paup v4 (Swofford

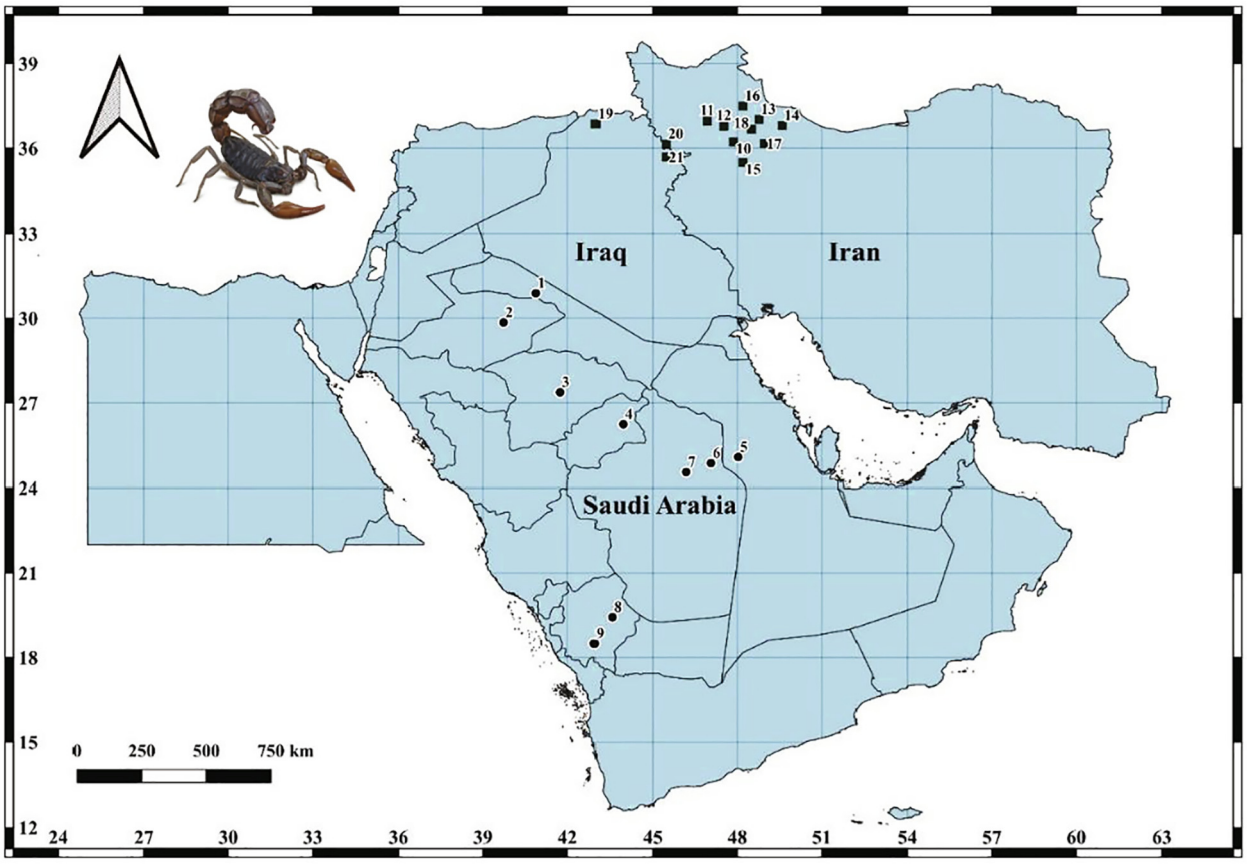

Fig. 1. Collection localities of *Androctonus crassicauda* samples from Saudi Arabia and GenBank sequences from Iraq and Iran that are given in Table 1.

Table 1
List of *Androctonus crassicauda* samples collected from Saudi Arabia used in this study and GenBank sequences, accession numbers of COI marker previously used in phylogenetic analysis.

No	*Species*	Location	Region	Ecogeoraphical region	Country	N	Lat.	Long.	Accession number	Authority
1	*A. crassicauda*	Arar	Northern Borders Province	**North Arabian Desert**	**Saudi Arabia**	3	30.88	40.87	–	This Study
2	*A. crassicauda*	Dumah Al Jandal	Al Jowf			3	29.84	39.73	–	This Study
3	*A. crassicauda*	Hail	Hail Province	**Central Arabian Desert**		3	27.37	41.73	–	This Study
4	*A. crassicauda*	Buraydah	Al Qassim			2	26.241	43.94	–	This Study
5	*A. crassicauda*	Khurais	Eastern Province	**Arabian Sand Desert**		2	25.07	48.02	–	This Study
6	*A. crassicauda*	Nazeem, east of Riyadh	Riyadh			1	24.86	47.06	–	This Study
7	*A. crassicauda*	Dhurma				1	24.54	46.17	–	This Study
8	*A. crassicauda*	Tathleeth	Aseer Province	**Southwestern Arabian Escarpment and Highlands**		2	19.42	43.56	–	This Study
9	*A. crassicauda*	Wadi Al Shiq				1	18.49	42.93	–	This Study
10	*A. crassicauda*	Mahneshan	Mahneshan	–	**Iran**	1	36.22	47.86	MH352603	Gene Bank
11	*A. crassicauda*	Sari_Aghol		–		1	36.95	46.93	MH352604	Gene Bank
12	*A. crassicauda*	Sahand-e Olya		–		1	36.77	47.52	MH352605	Gene Bank
13	*A. crassicauda*	Darram,	Zanjan Province	–		1	37.02	48.77	MH352606	Gene Bank
14	*A. crassicauda*	Chavarzagh	Zanjan Province	–		1	36.80	49.66	MH352607	Gene Bank
15	*A. crassicauda*	Taroom,	Sansooz	–		1	35.49	48.20	MH352608	Gene Bank
16	*A. crassicauda*	Zanjan	Zanjan Province	–		1	36.69	48.50	MH352609	Gene Bank
17	*A. crassicauda*	Doasb,	Zanjan Province	–		1	36.16	48.95	MH352610	Gene Bank
18	*A. crassicauda*	Daneshgah	Zanjan Province	–		1	36.67	48.50	MH352611	Gene Bank
19	*A. crassicauda*	Sardasht,	West Azerbaijan Province	–		1	36.14	45.47	MK814934	Soltan et al., 2021
20	*A. crassicauda*	Sardasht,	West Azerbaijan Province	–		1	35.68	45.19	MK814933	Soltan et al., 2021
21	*A. crassicauda*	–	–	–	**Iraq**	1	36.86	42.98	MT229840	Gene Bank

2001) with heuristic searches using stepwise addition followed by tree bisection reconnection (TBR) branch swapping (Swofford et al. 1996). In all alignments, gaps were treated as missing characters. Confidence within the nodes was evaluated using 1000 bootstrap replicates (Felsenstein 2002) with random addition of taxa. MrModeltest 2.3 (Nylander 2004) was used to select the best-fit models of nucleotide evolution supported by Akaike information criterion (AIC) (Akaike 1973). The geographic structure was inferred using Bayesian inference (BI) implemented with MrBayes 3.1.2 (Ronquist et al. 2012). Analyses were run for one million generations and the output parameters were visualized to determine stationarity and convergence using Tracer 1.4 (Rambaut & Drummond, 2007).

3. Results

This study comprised 38 sequences with 556 nucleotide sites of *Androctonus* species and the applied outgroup. In total 384 (69.0%) nucleotide sites were polymorphic and 104 (18.7%) were parsimony informative. Within the in-group, 58 (10.4%) sites were polymorphic and 45 (8.09%) were parsimony informative. Tamura and Nei (1993) genetic distances between the Arabian *A. crassicauda* populations ranged from d = 0.01 to 0.08, and from d = 0.10 to 0.11 between *Androctonus* species in-group sequences (Table 2).

The phylogenetic tree topologies recovered by the analysis of the COI data set, from the MP, NJ, and BI analysis, were the same in outlining two major clades of *A. crassicauda* (Figs. 2, 3, 4). The first clade includes the population of the North Arabian Desert (NAD), and those of Iraq and Iran. This first clade is split further into two well-supported subclades. The first includes the population of the Northern part of Saudi Arabia, represented by six specimens from the Northern Border Province and Al Jowf populations. The second subclade consists of one specimen from Iraq and eleven from northern Iran. The second clade represents the remaining populations of the Central Arabian Desert (CAD), Arabian Sand Desert (ASD) and the Southwestern Arabian Escarpment and Highlands (SAEH) grouped as a sister phylogroup.

4. Discussion

Androctonus crassicauda is one of the medically important scorpion species occurring in the Middle East. Šmíd et al., (2021) defined six different ecogeographical regions across Saudi Arabia, including the North Arabian, Central Arabian, the Arabian Sand and Tihama Deserts, as well as the Red Sea Shrublands, and Southwestern Arabian ridge and highlands. Our study of *A. crassicauda* scorpion populations found a relatively high population genetic diversity, retrieving three main lineages in ten investigated populations.

Table 2
Genetic distance based on mitochondrial COI sequences between different *Androctonus* genus. Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the Tamura-Nei model. SA = Saudi Arabia.

Populations/ Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. Northern_Borders_Province, SA		0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
2. Al_Jowf, SA	0.01		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
3. Hail_Province, SA	0.07	0.07		0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
4. Al_Qassim, SA	0.07	0.07	0.02		0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
5. Eastern_Province, SA	0.07	0.07	0.02	0.02		0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
6. Riyadh_Province, SA	0.07	0.07	0.01	0.02	0.01		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
7. Aseer_Province, SA	0.08	0.07	0.01	0.02	0.02	0.02		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
8. Iran	0.05	0.06	0.08	0.08	0.08	0.08	0.08		0.01	0.01	0.01	0.01	0.01	0.01	0.01
9. Iraq	0.05	0.05	0.08	0.08	0.08	0.07	0.08	0.03		0.01	0.01	0.01	0.01	0.01	0.01
10. *A. liouvillei*	0.10	0.10	0.11	0.11	0.11	0.11	0.11	0.09	0.09		0.01	0.01	0.01	0.01	0.01
11. *A. gonneti*	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.09	0.09	0.08		0.01	0.01	0.01	0.01
12. *A. australis*	0.10	0.10	0.10	0.11	0.10	0.10	0.10	0.10	0.09	0.11	0.10		0.01	0.01	0.01
13. *A. mauritanicus*	0.10	0.10	0.09	0.10	0.10	0.09	0.10	0.10	0.10	0.09	0.11	0.08		0.01	0.01
14. *A._amoreuxi*	0.10	0.10	0.11	0.12	0.11	0.11	0.11	0.11	0.10	0.07	0.09	0.10	0.10		0.01
15. *A. bicolor*	0.11	0.11	0.11	0.11	0.11	0.10	0.11	0.12	0.11	0.08	0.10	0.10	0.09	0.10	

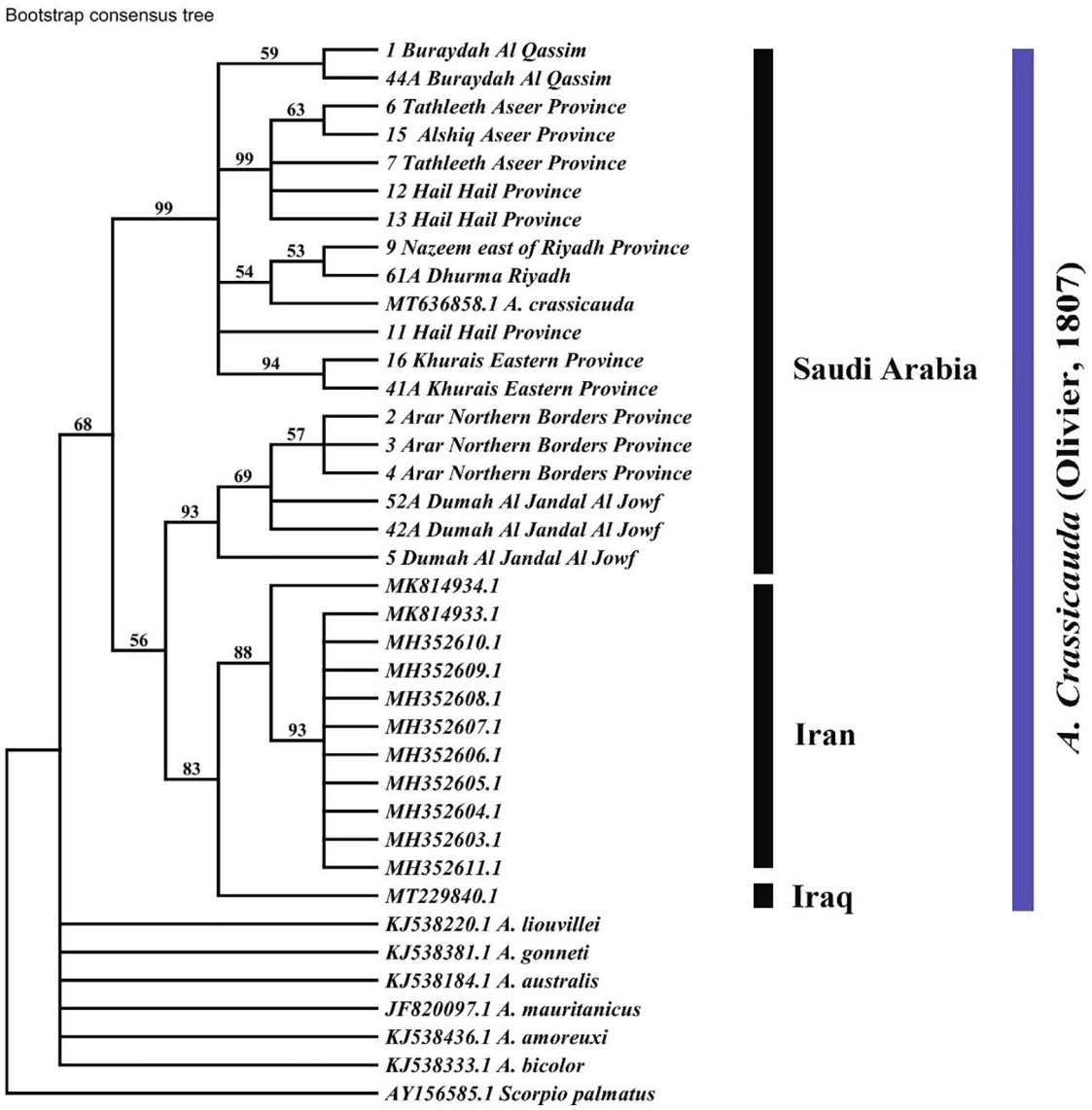

Fig. 2. Maximum-parsimony phylogenies of *Androctonus crassicauda* sequences fragment of the COI gene. Numbers above the branches represent bootstrap values calculated with 1000 replicates.

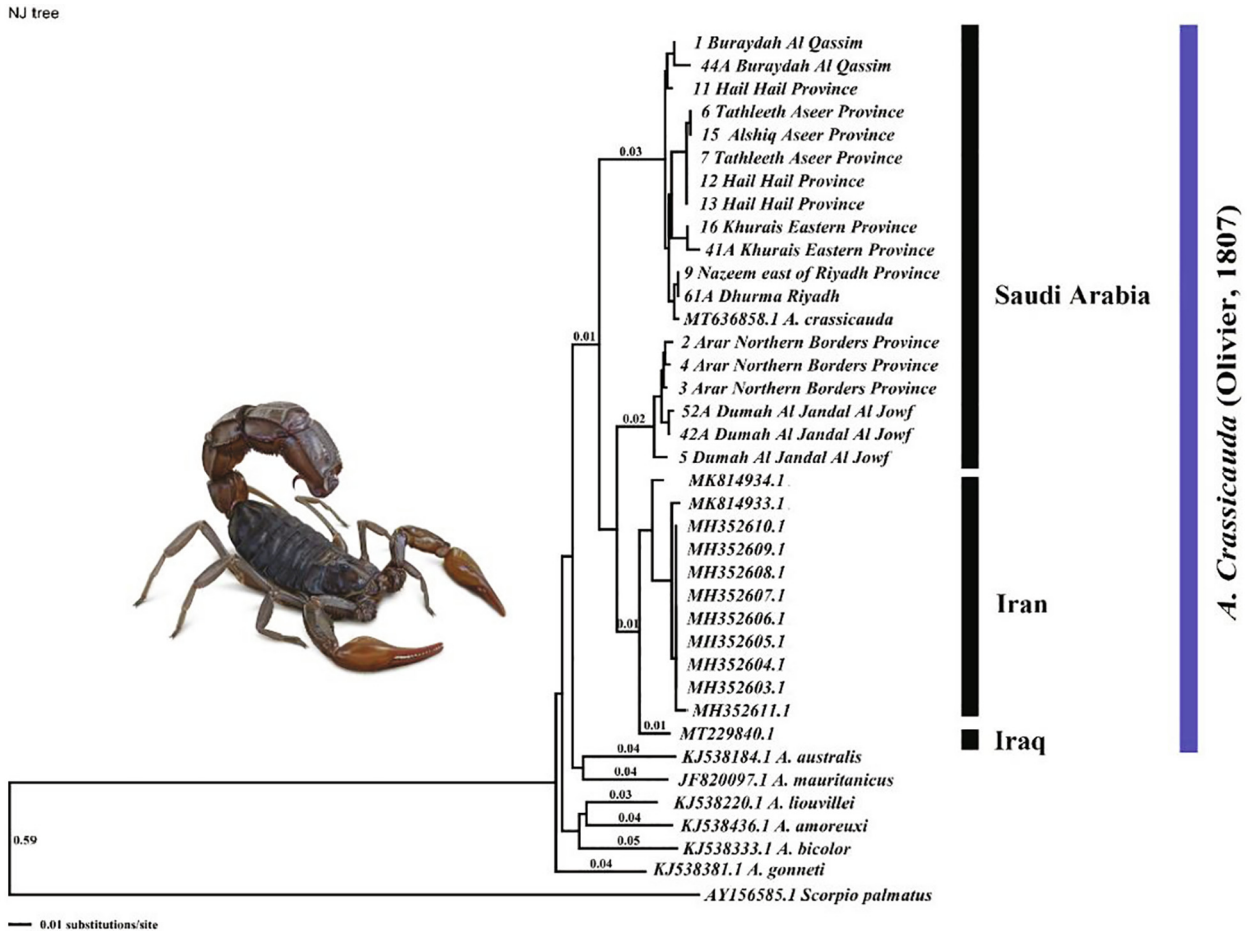

Fig. 3. Neighbor-joining phylogenies of *Androctonus crassicauda* sequences fragment of the COI gene. Numbers above the branches represent distance values.

Previously, several studies reported similar results conducted with the same and allied genera, such as *Androctonus* Ehrenberg, 1828, *Buthus* Leach, 1815, *Buthacus* Birula, 1908, *Hottentotta* Birula, 1908, *Leiurus* Hemprich and Ehrenberg (1829) and *Scorpio* L., 1758 (Gantenbein and Largiadèr 2000; Froufe et al. 2008; Ben Othmen et al., 2009: Ozkan et al., 2010; Sousa et al. 2011; Coelhoa et al., 2014; Toprak et al., 2019; Alqahtani and Badry 2020a, b; Sarhan et al., 2020; Toprak et al., 2019). The phylogenetic analysis of the current populations of *A. crassicauda* has divided into three monophyletic well supported clades was unexpected. The first encompasses five Saudi Arabian populations grouped in a sister clade. These populations are from three different ecogeographical regions including the Central Arabian desert (CAD), the Arabian Sand desert (ASD), and the Southwestern Arabian Escarpment and Highlands (SAEH). The second lineage encompasses two Saudi Arabian populations from two different localities of the North Arabian Desert (NAD). The third lineage consists of twelve specimens from Iraq and Iran (Fig. 1, Table 1).

Despite that the genetic distance between Saudi Arabian clades is relatively high, this may indicate that *A. crassicauda* represents two cryptic species. The genetic distances between *A. crassicauda* obtained from the North of Arabian desert (NAD) and those obtained from other ecogeographical regions of Saudi Arabia ranged from 7 to 8% (Table 2). Also, the distance between the North Arabian Desert (NAD) populations and those from Iraq and Iran ranged from 5 to 6% (Table 2). Due to the shortage of clear morphological features used in the traditional taxonomy in many scorpions' taxa, the occurrence of cryptic species seems to be common (Gantenbein, et al., 2000). Furthermore, analysis of genetic variations between populations of *Scorpio fuliginosus* (Pallary, 1928) from Morocco identified genetically distinct lineages (Froufe et al., 2008). The high intraspecific genetic variability within *Androctonus* scorpions may be due to prominent geographical features, which contribute to increasing scorpion inclination for diversification in association with long-term episodes of geomorphological changes and climatic changes. The divergence between the Central and Southwestern Saudi Arabia from the north and Iraqi- Iranian populations were probably associated with the Taurus-Zagros Mountains and the Saharo-Arabian developing desert belt, which may act as a biogeographic filter (Jacobs et al., 1999). Regardless of the morphological revision, our study seems to provide strong evidence for the existence of three separate lineages of *A. crassicauda*, which could be confirmed by further study. Generally, identification of hidden variation in scorpion species is essential, not only to revise taxonomy, but also because the many studies assessing the biochemical nature of scorpion venoms require precise species determination (Kharrat et al. 1997).

Further taxonomic and morphometric studies should be undertaken among the different populations of *A. crassicauda* in the Arabian Peninsula to reveal other differential characters, and perhaps describe new species.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

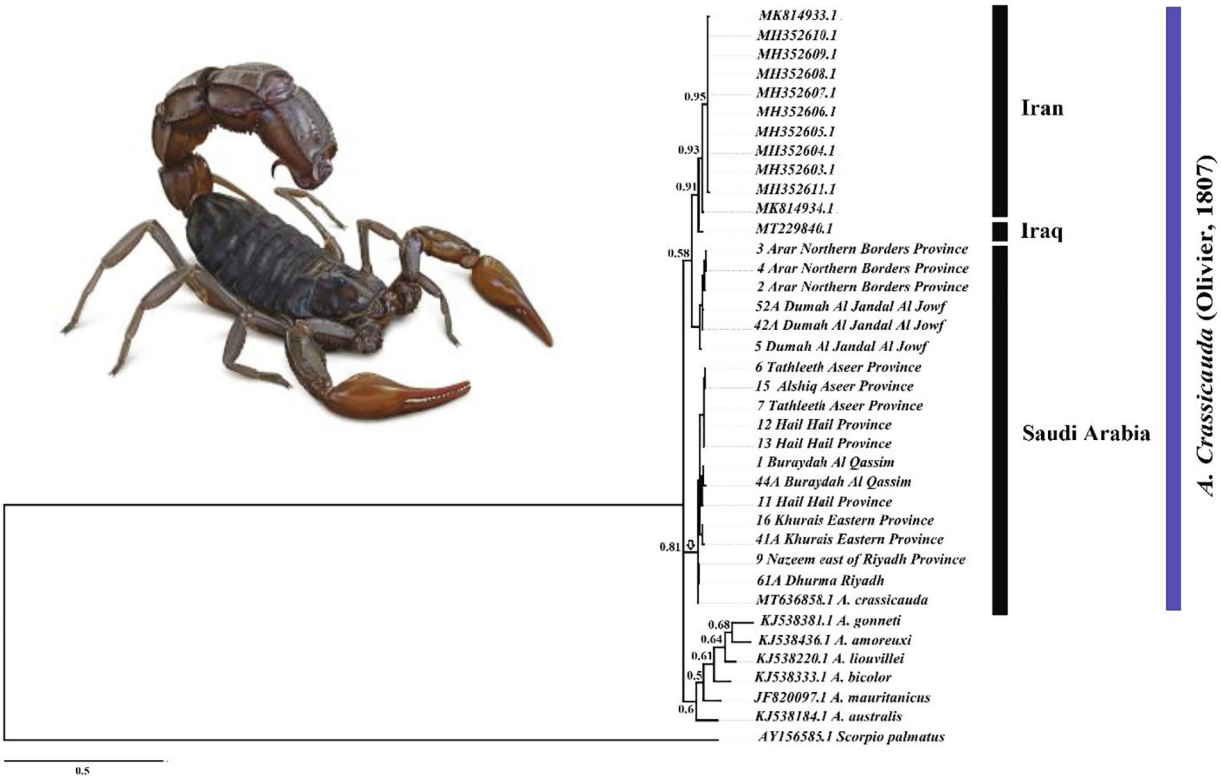

Fig. 4. Bayesian inference tree phylogenies of genus *Androctonus crassicauda* sequences fragment of the COI gene. Numbers above the nodes represent the posterior probabilities.

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