RESEARCH ARTICLE

# COI-based molecular phylogeny of some Buthidae scorpions from Egypt

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**Buthidae family is a scorpion family distributed throughout the world, especially in North Africa. The individual and geographical variability of this family members is associated with evolutional genetic development reflected in their venoms even in the same species. Intragenic differences can be a result of genetic and environmental factors. DNA barcoding is a system that, based on the conserved DNA sequences, helps in differentiating the different species and same species in different habitat. This study aimed on using the mitochondrial cytochrome c oxidase 1 (COX1 or CO1 or COI) to obtain molecular identification information about the taxonomic status of a set of scorpion species (*Androctonus crassicauda*, *Androctonus bicolor*, *Androctonus amoreuxi*, *Leiurus quinquestriatus*, and *Buthacus arenicola*) collected from Egypt. COI gene is slowly evolving comparing to other protein-coding mitochondrial genes. Therefore, it is widely used for estimating molecular phylogenies. Also, COI gene represents one of the largest sequence data sets generated from any group for phylogenetic study and fulfills the phylogenetic accuracy putative. The sequencing data of the cytochrome oxidase 1 gene were applied to provide information for better understanding of the intraspecific variation, evolution, and genetic distance between these species. COI data from the Egyptian *A. australi*, and those of *L. quinquestriatus* from Sudan, previously reported in Egypt, had been included for genetic comparison. The results revealed that the high genetic diversity was found among *A. amoreuxi* and *A. australis*. There is no detectable genetic variation between the Egyptian samples of *L. quinquestriatus* and those isolated from Sudan. This study helped us to understand the current evolution of these six scorpions on the genetic level and gave us better understanding of differences of scorpion envenomation (SE) of the same species in different localities.**

**Keywords:** cytochrome oxidase 1; phylogeny; taxonomy; mitochondrial DNA; scorpions; Buthidae.

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## Introduction

Buthidae family represents about 81 (48.8%) of the 166 scorpion genera that have been described and about 700 (46.7%) of the approximately 1,500 described scorpion species. This family includes species of the genera *Androctonus*, *Leiurus*, and *Buthacus*. All scorpions possess venoms, but about 30 to 50 are medically significant species that are members of the family Buthidae [1]. Buthidae is known as bark scorpions and thick-tailed scorpions and is also considered as one of the most dangerous groups in the world. They are found throughout the arid and semi-arid regions of the Middle East and Africa [2]. Scorpions are one the most ancient animals, which have survived over 400 million years. During this long evolutionary time, they have largely preserved their morphology, so that they earned the label of living fossils [3–6].

The four species including *Androctonus amoreuxi*

(Audouin, 1826), *A. australis* (Linnaeus, 1758), *A. bicolor* (Ehrenberg, 1828), and *A. crassicauda* (Olivier, 1807) were reported as the most medically important groups of scorpions [7]. *Leiurus quinquestriatus* is the most common toxic species and it is found in Egypt [8]. Recently, scorpion taxonomy and systematics have shown a real need for revision with different tool because the morphological features are thought not sufficient to rely on due to some species delineation. Within the family Buthidae, there are morphological similarities and immense intraspecific morphological diversity among species, thus identification and delimitation are relatively difficult [9]. Updating classification of animals, including scorpions, not only helps better understanding of taxonomic position but also helps to understand scorpion envenomation (SE). This also supports the medical preparation of suitable anti-venom in a certain area, which should be specific, otherwise it will not be effective [10]. In Upper Egypt, scorpion envenomation is an agonizing problem, especially in rural areas [11]. That leads to the conclusion that clinical manifestations of scorpion sting and the final outcome varies according to the geographical site and predominant scorpion species in this area [12].

Many researchers used mitochondrial DNA (mtDNA) as a source of molecular markers for studying population genetic structure, molecular diversity, evolution properties, and the phylogeography of various scorpion species because it possesses haploid maternal inheritance and high mutation rate [13–16]. The mitochondrial gene, cytochrome c oxidase I (COX1 or CO1 or COI), has a DNA sequence that can be used as taxon 'barcodes' and serves as the core of a global bio identification system [17]. This study was designed to use COI gene from 5 different species of scorpions from Egypt including *Androctonus amoreuxi*, *Androctonu bicolor*, *Androctonus crassicauda*, *Buthacus Arenicola*, and *Leiurus quinquestriatus* to study the genetic variations and molecular phylogeny among Egyptian scorpions from different localities.

## Materials and methods

### Sample collection

Five Egypt scorpion species of Buthidae family including *Androctonus amoreuxi* (A.AM.Eg), *Androctonus bicolor* (BI.Eg), *Androctonus crassicauda* (A.CR.Eg), *Buthacus arenicola* (B.AR.Eg), and *Leiurus quinquestriatus* (L.Q.Egy) were collected from Baltim, Matruh, southern range of Sina peninsula, El Maghara in North Sina Peninsula, and Aswan, respectively. Four samples of each species were collected except *Leiurus quinquestriatus* with 10 samples. Samples then were kept in separated small containers till dissection time. Muscle tissues of the legs were dissected for the DNA extraction.

### DNA extraction

DNA extraction was performed from fresh and preserved muscle tissues by using QIAamp DNA Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's recommendations. Briefly, tissues were isolated, minced in ALT buffer and then proteinase K for lysis step. For precipitation step AL buffer and 99% ethanol were used. Finally, AW1 and AW2 were used in purification step. The samples were stored in AE buffer at 4°C until use.

### Mitochondrial DNA fragment amplification

Polymerase chain reaction (PCR) was used to amplify the mtDNA fragment of COI by using *Drosophila yakuba* (GenBank accession number: X03240) forward (LCO1490) (GGTCAACAAATCAT CATAAAGATATTGG and reverse (HCO219) (TAA ACTTCAGGGTGACCAAAAAATCA) primers [18]. PCR was performed in final volume of 25 µL with 10 pmol (1.0 µL) of each primer, 12.5 µL of PCR master mix (Dongshing, Guangzou, Guangdong, China), 1.0 µL of purified DNA, and 9.5 µL of water. Amplification condition was 94°C for 4 min followed by 35 cycles of 94°C for 50 s, 53°C for 50 s, and 72°C for 60 s, and then 72°C for 5 min. The amplification was done by using LabCycler 48 (SensoQuest GmbH, Göttingen, Germany) and was confirmed by gel electrophoresis on 2% agarose gel. The PCR products were kept and subjected for DNA sequencing.

**Table 1.** COI data that was retrieved from GenBank along with the isolated species and the outgroup.

| Taxon | Description | GenBank Accession number | Country |
|---|---|---|---|
| *Androctonus amoreuxi* | A.AM.Egy | MT629926 | Egypt |
| *Androctonus amoreuxi* | A.AM1 | KJ538478.1 | Egypt |
| *Androctonus amoreuxi* | A.AM2 | KJ538480.1 | Egypt |
| *Androctonus amoreuxi* | A.AM3 | KJ538483.1 | Egypt |
| *Androctonus australis* | A.AU1 | KJ538452.1 | Egypt |
| *Androctonus australis* | A.AU2 | KJ538455.1 | Egypt |
| *Androctonus australis* | A.AU3 | KJ538462.1 | Egypt |
| *Androctonus bicolor* | B.BI.Egy | MT636859 | Egypt |
| *Androctonus bicolor* | A.BI1 | KJ538333.1 | Egypt |
| *Androctonus bicolor* | A.BI2 | KF548120.1 | Egypt |
| *Androctonus crassicauda* | A.CR.Egy | MT636858 | Egypt |
| *Buthacus arenicola* | B.AR.Egy | MT636861 | Egypt |
| *Leiurus quinquestriatus* | L.Q.Egy | MT636860 | Egypt |
| *Leiurus quinquestriatus* | L.Q1 | KX648420.1 | Sudan |
| *Leiurus quinquestriatus* | L.Q2 | KX648421.1 | Sudan |
| *Leiurus quinquestriatus* | L.Q3 | JQ514258 | Egypt |
| *Didymocentrus kraus* | D.krausi outgroup | KM514633.1 | Central American |

**DNA sequencing**

PCR amplicons sequencing was performed by Sanger method according to the standard protocol. Samples were sequenced by Macrogen company (Seoul, Republic of Korea).

**DNA sequences data retrieval and analysis**

All COI gene sequences of the collected species from Egypt were retrieved from the GenBank along with 2 sequences of *Leiurus quinquestriatus* originally from Sudan because they both were labeled as Egyptian scorpions. All sequences accession numbers were listed in table 1. Sequence alignment was carried out by using Molecular Evolutionary Genetics Analysis (MEGA) X [19] (https://www.megasoftware.net) with clustalW. The maximum likelihood was performed as 5,000 replicas with substitution model GTM+I. The genetic distances between clades and within clades were also performed by using MEGA X and DnaSP v5 [20] (http://www.ub.edu/dnasp) to estimate nucleotide divergence and polymorphism.

## Results

**Nucleotide frequencies**

The sequence analysis revealed that the range of A+T content among studied species was between 58% to 59.3% while the range of G+C content was between 40.5% to 41.5% (Table 2). The highest A+T content was *A. crassicauda* and the lowest one was *A. amoreuxi*. The lowest G+T content was *A. crassicauda* and the highest one was *A. amoreuxi*.

**Sequenced data analysis**

The COI gene sequences of 16 scorpion species and one outgroup were included for sequence comparing and aligning studies (Table 1). The dataset consisted of an alignment of 662 base pairs with 587 sites were applied in this study after excluding 75 sites with gaps or missing data. The applied dataset included 425 invariable (monomorphic) sites and 162 variable (polymorphic) sites with 116 parsimony informative sites. The sequence analysis results demonstrated 162 polymorphic (segregating) sites, 201 mutations, 46 singleton variable sites, and 14 haplotypes. The nucleotide diversity (Pi) was 0.09670 and haplotype (gene) diversity (Hd) was 0.978.

**Table 2.** The average nucleotide frequencies of COI sequences from five isolated scorpion species in Egypt.

| | T(U)% | A% | C% | G% | A+T Content (%) | G+C Content (%) | Total base pair length |
|---|---|---|---|---|---|---|---|
| *A. bicolor*. Egy | 40.7 | 18.48 | 13.03 | 27.7 | 59.18 | 40.73 | 606.0 |
| *L. quinquestriatus*. Egy | 40.7 | 17.80 | 13.00 | 28.0 | 58.50 | 41.00 | 609.0 |
| *B. arenicola*. Egy | 40.8 | 18.00 | 13.00 | 27.8 | 58.80 | 40.80 | 617.0 |
| *A. amoreuxi*. Egy | 41.0 | 17.00 | 12.80 | 28.7 | 58.00 | 41.50 | 609.0 |
| *A. crassicauda*. Egy | 40.5 | 18.80 | 13.00 | 27.5 | 59.30 | 40.50 | 600.0 |
| Average | 40.7 | 18.00 | 13.00 | 27.9 | 58.70 | 40.90 | 608.2 |

**Table 3.** The mean of interspecies genetic distance and P-distance of COI gene.

| | Distance between clades | | | | | | | | | Within clades | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | | 0.01146 | 0.01219 | 0.012017 | 0.01227 | 0.01251 | 0.01212 | 0.01201 | 0.01558 | 0.0036 | 0.0018 | **A** |
| **B** | 0.08897 | | 0.01253 | 0.012464 | 0.01253 | 0.01289 | 0.01269 | 0.01228 | 0.01563 | 0.0044 | 0.0022 | **B** |
| **C** | 0.09893 | 0.10553 | | 0.012207 | 0.01219 | 0.01219 | 0.01225 | 0.01246 | 0.01577 | 0.0000 | 0.0000 | **C** |
| **D.1** | 0.09901 | 0.10341 | 0.10192 | | 0.00000 | 0.01256 | 0.01250 | 0.01227 | 0.01535 | n/c | n/c | **D.1** |
| **D.2** | 0.10021 | 0.10147 | 0.10017 | 0.00000 | | 0.01252 | 0.01255 | 0.01252 | 0.01535 | n/c | n/c | **D.2** |
| **E** | 0.11010 | 0.11481 | 0.10967 | 0.10815 | 0.10667 | | 0.00602 | 0.00631 | 0.01496 | 0.0000 | 0.0000 | **E** |
| **F.2** | 0.10339 | 0.10981 | 0.10785 | 0.10478 | 0.10592 | 0.02411 | | 0.00297 | 0.01491 | 0.0016 | 0.0016 | **F.2** |
| **F.1** | 0.10095 | 0.10396 | 0.11010 | 0.10231 | 0.10518 | 0.02451 | 0.00576 | | 0.01505 | n/c | n/c | **F.1** |
| **O. group** | 0.18737 | 0.18437 | 0.19446 | 0.17707 | 0.17500 | 0.17175 | 0.17049 | 0.16887 | | n/c | n/c | **O. group** |
| | **A** | **B** | **C** | **D.1** | **D.2** | **E** | **F.2** | **F.1** | **outgroup** | **p-distances.** | **St. err.** | |

**Table 4.** The numbers of nucleotide sequences, mutations, average nucleotide differences, nucleotide diversity, and separating positions (polymorphic) in the six clades that were presented in the COI ML tree.

| | A | B | C | D | E | F2 |
|---|---|---|---|---|---|---|
| **Number of sequences** | 4 | 3 | 2 | 2 | 2 | 2 |
| **Total number of mutations** | 4 | 4 | 0 | 0 | 0 | 1 |
| **Number of polymorphic sites** | 4 | 4 | 0 | 0 | 0 | 1 |
| **Average number of nucleotide differences, (k)** | 2.167 | 2.667 | 0.000 | 0.000 | 0.000 | 1.000 |
| **Nucleotide diversity, (Pi)** | 0.00348 | 0.00440 | 0.00000 | 0.00000 | 0.00000 | 0.00165 |

## Overall phylogeny

*Androctonus amoreuxi* and *Androctonus australis* were monophyletic as represented in the maximum likelihood (ML) tree (Figure 1). Six clades were all identified with high values of bootstrap support (ML). Nevertheless, the relationships between some species could not always be resolved. *Androctonus amoreuxi* isolated from Baltim clustered with all *A. amoreuxi* species and formed a monophyletic group. *Androctonus australis* also formed a monophyletic unit. In this analysis, clade C contained two *Androctonus bicolor* specimens. However, the sample isolated from this study wasn't clustered with them and was found in clade D with *Androctonus crassicauda* as both were isolated from Matruh and Southern range of Sina Peninsula, respectively. *Leiurus quinquestriatus* was presented in clades E and F which were sister clades. Clade E species originated from Sudan but were labeled as Egyptian scorpions in GenBank. Therefore, they were included in this analysis. Clade F contained two subclades F1 and F2. F1 included *Buthacus arenicola* isolated from El Maghara in North Sina Peninsula. F2 included *L. quinquestriatus* from Aswan and other Egyptian *L. quinquestriatus*. All the clades demonstrated the highest support values (ML = 100) except clade F (ML = 94).

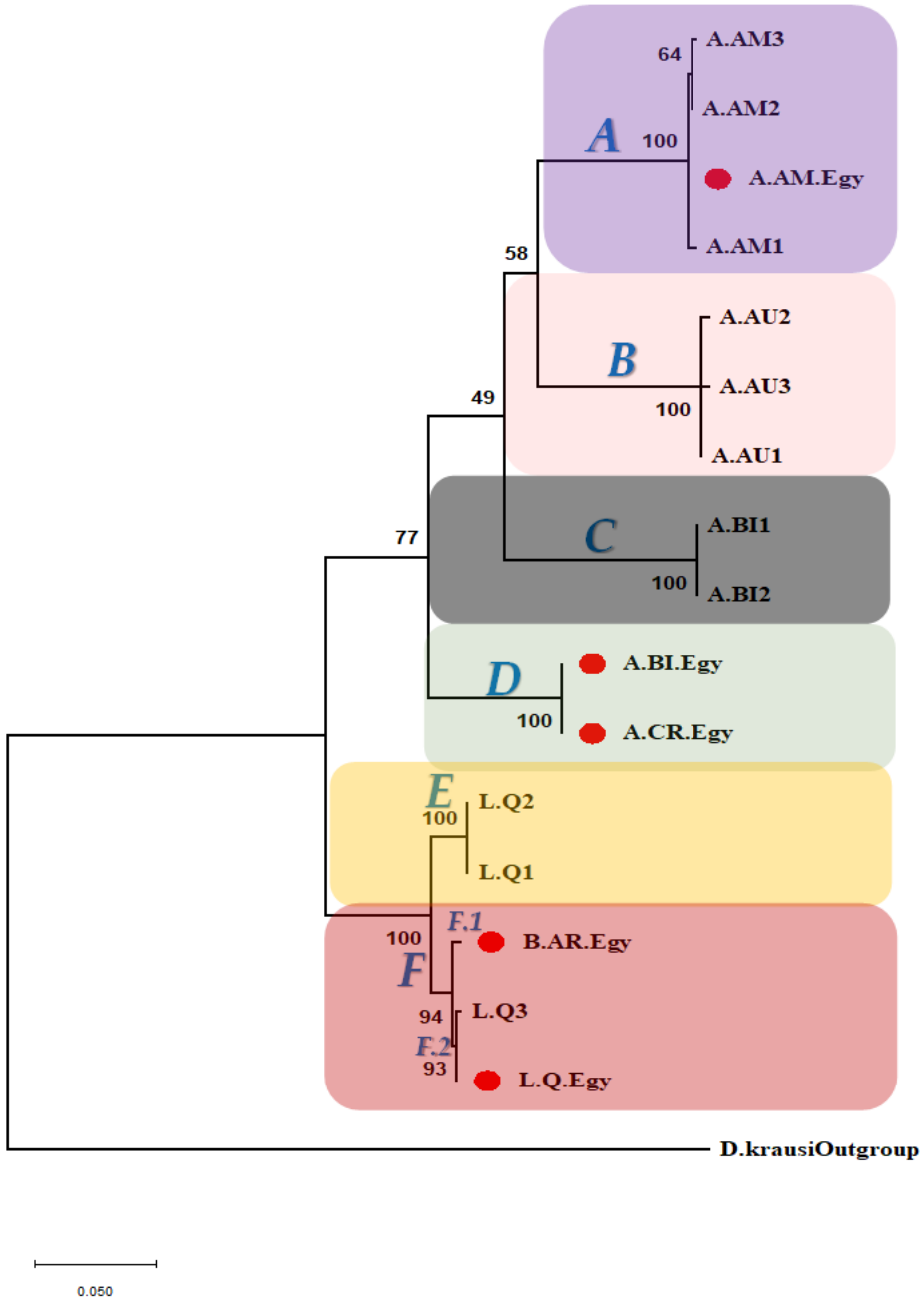


**Figure 1.** The phylogenetic maximum likelihood (ML) 50% majority-rule consensus tree of COI gene. There were 6 main clades. The red label showed the samples isolated from this study. Branches indicated bootstrap values calculated with 5,000 replicates.

**Genetic distance**

The genetic distance between all clades is ranging from 0.5% to 11% (Table 3). The lowest genetic distance was between the two subclades of clade F (F1 and F2), while the highest genetic distance appeared between clade E and A, as well as between clade F1 and C. The highest P-distance within a clade is 0.4% which appeared in clade B while the lowest one was in clade C and E.

**DNA polymorphism and divergence**

The number of nucleotide sequences, mutations, average nucleotide differences, nucleotide diversity, and separating positions (polymorphic)

were shown in Table 4. The six clades were presented in the COI ML tree (Figure 1). The results represented total 15 specimens divided as clade A (n=4), B (n=3), C (n=2), D (n=2), E (n=2), and subclade F2 (n=2).

## Discussion

Scorpions of Egypt are widely distributed and known for their toxicity. Several species found currently near the Mediterranean coastline are *Androctonus australis*, *Androctonus bicolor*, *Buthacus* sp., *Orthochirus innesi* (Buthidae), and *Scorpio maurus* (Scorpionidae). The first two buthids are considered among the most dangerous of scorpions. They are always found far from the sea, and west of Alexandria. Natural habitats have considerably changed at the Egyptian coastline of the Nile Delta and in the Alexandria area in the last 2,300 years due to the erosion that threatens the coastal cities. These changes during the 19th and 20th centuries continued and increased as a result of a natural decrease of river controls and the Nile's discharge. The change in habitat can cause changes in gene flow and diversification among scorpion populations [21–23]. In this study, we applied COI gene as a phylogenetic marker to reveal the diversification among the Egyptian scorpions because that hardly to be revealed by nuclear gene as stated before [24]. *Androctonus* species has extraordinary polymorphism within their nuclear genes so that it is difficult to distinguish the species. However, many previous studies applied the mitochondrial DNA genes to detect the divergence between scorpions populations [16, 25, 26]. Notably, limited studies have focused on the Egyptian scorpion phylogeny. Only two species have been studied *L. quinquestriatus* and *B. arenicola* [21, 27, 28]. In this study, 6 species of Egyptian scorpion were included to identify the divergence among their MtDNA lineages.

*A. crassicauda* is very rare in Egypt and was found only in South Sinai [29]. There is no record in the GenBank regarding COI gene sequences from the Egyptian species of scorpion. *A. crassicauda* could be overlapped with *A. bicolor* because of their black color. However, they show minor similarity according to their protein profile (S = 0.26) [29]. However, our result showed no genetic diversity among them. Based on the sequence similarity, Iranian scorpion *A. crassicauda* has a close relationship with *A. bicolor* from southern Israel in their mammalian neurotoxin [30].

The p-distance between the Egyptian species *B. arenicola* and *A. crassicauda* is 10.59% when using the COI gene as the marker. According to the report of Alqahtani and Badry, the genetic distance between the two species was 22% by using 16S rRNA as the marker [28]. On the other hjand, the *A. crassicauda* species they used in the study was collected from Turkey (GenBank accession number: FJ217735.1). Both results gave the assumption that *A. crassicauda* species from Egypt and Turkey could have diverged. There is no available data about B. arenicola COI mitochondrial gene sequences. Therefore, the analysis for *B. arenicola* identification is limited. However, Alqahtani and Badry confirmed that there was no genetic distance among *B. arenicola* from North Saini and those from northwestern Egypt [28]. *A. crassicauda* from Turkey (GenBank accession number: AJ277598.1) has a high genetic distance valued of 53% or 51% from Egyptian *L. quinquestriatus* when 16S rRNA or mitochondrial gene markers were used, respectively [21, 27], while the Egyptian *A. crassicauda* showed low P-distance value in a range of 10.59% to 10.66 % from clade E and subclade F2, in which both clades included *L. quinquestriatus* from Egypt and Sudan. There was no divergence observed between the *L. quinquestriatus* species that occupied African part of Egypt, while the high divergence was observed between *L. quinquestriatus* from Sina part and from Africa part (8% to 0%) if using 16S RNA marker. This finding may indicate that *L. quinquestriatus* represents two species as reported before [21, 27], which also supports our results that the distance between E clade and F2 subclade was 2% and the distances within clade

were E = 0 and F2 = 0.1%. The nucleotide diversity (Pi) was 0.165% and the mean of nucleotide differences (K) was 1 between the Egyptian species. All of *L. quinquestriatus* used in this analysis were African species.

Ben Ali, *et al*. stated that *A. aeneas* (*A. bicolor*) might be a race of *A. amoreuxi* [24]. However, according to Ben Othmen, *et al*., the p-distance between both species were ranging from 10% to 13.6% [31]. Our results demonstrated 9.9% genetic distance value, therefore, both results rejected the conclusion of Ben Ali, *et al*. [24]. In the similarity index of different electropherograms of protein subunits, *A. amoreuxi* showed high similarity with *A. bicolor* (S = 0.83) and *A. australis* (S = 0.9) [29], which indicated that *A. bicolor* showed high similarity with *A. amoreuxi* and was contrast to the genetic distance value results of this study. For *A. amoreuxi* and *A. australis*, the genetic distance value between their clades (A and B) was 8.8%. Thus, they are genetically closer than Tunisian *A. amoreuxi* and *A. australis* species that their genetic distance ranges from 15.9% to 21.5% [31]. Clade A showed 4 species of Egyptian *A. amoreuxi* with nucleotide diversity (p-distance) 0.3%. Clade B contained 3 species of *A. australis* with high nucleotide diversity 0.4% and high nucleotide differences (K = 2.66). However, the genetic variation that was represented in the study in the scorpion populations could be from genetic drift or inbreeding in scorpion populations [32].

In summary, the molecular and phylogenetic analysis should be applied more in Egypt to investigate the relative, demographic, and genetic structure variations of various species of scorpions in different regions to detect scorpion diversity.

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