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Reconstructing Ancestral Connections in the Sahara Using the COI Gene: The Role of Western Libyan Cerastes cerastes in Biodiversity Conservation and Forensic Identification

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إعادة بناء الروابط السلالية في الصحراء الكبرى باستخدام جين *COI*: دور أفعى القرون الليبية الغربية (*Cerastes cerastes*) في حفظ التنوع الحيوي والتعرّف الجنائي

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Abstract:
North African horned vipers (***Cerastes cerastes***) represent a widespread but poorly understood species complex across Saharan and Sahelian regions. Libya, despite its central geographic position, remains critically under-sampled in molecular phylogenetic studies of this group. Here we present the mitochondrial COI barcode sequence (621 bp) of a *Cerastes cerastes* specimen from the Nalut region (western Libya, near the Tunisian border). We compared this sequence to 11 conspecific sequences from Egypt, Chad, Niger, Mauritania, Morocco, and two outgroup species (*Cerastes gasperetti*, *Cerastes vipera*) using Maximum Likelihood (ML), Neighbor-Joining (NJ) phylogenetic reconstruction, Median-Joining network analysis, and pairwise genetic distances under the Kimura-2-parameter (K2P) model. Our results reveal that the Libyan Nalut specimen forms a strongly supported clade (bootstrap 99–100%) with populations from Morocco (Figuig and Oued Lakhchab) and Mauritania (Tiris Zemmour, Tagant, Trarza, Dakhlet-Nouadhibou), showing close genetic affinity (mean K2P distance ~0.032–0.034). In contrast, the Libyan haplotype is genetically distant from Egyptian and Chadian populations (mean distance ~0.045–0.063), suggesting a phylogeographic break coincident with the Libyan Desert and the Nile River corridor. The Median-Joining network demonstrated a novel haplotype in the Nalut region, which could indicate to cryptic diversity and potential local endemism. These findings could have direct implications for snake conservation in Libya, taxonomic revision of the *Cerastes cerastes* complex, and forensic identification. We strongly recommend expanded sampling across Libya's diverse ecoregions to fully document the hidden genetic diversity in this understudied North African biodiversity hotspot.

Keywords: *Cerastes cerastes*, mitochondrial DNA, COI barcode, Libya, Nalut, Sahara biogeography, cryptic diversity, forensic herpetology.

الملخص
تمثّل أفعى القرون في شمال أفريقيا (*cerastes Cerastes*) مجموعة واسعة الانتشار، لكنها لا تزال قليلة التمثيل في الدراسات الوراثة التطورية، ولا سيما داخل ليبيا. تهدف هذه الدراسة إلى تحليل تسلسل جين الميتوكوندريا COI (طول 621 زوج قواعد) لعينة من Cerastes cerastes مأخوذة من منطقة نالوت (غرب ليبيا، قرب الحدود التونسية)، ومقارنته مع 11 تسلسلًا من النوع نفسه من مصر وتشاد والنيجر وموريتانيا والمغرب، إضافة إلى نوعين خارج المجموعة هما *gasperetti Cerastes* و*vipera Cerastes*. استُخدمت طرق Maximum Likelihood وNeighbor-Joining، وتحليل شبكة Median-Joining، وحساب المسافات الوراثية الزوجية وفق نموذج Kimura-2-parameter (K2P). أظهرت النتائج أن عينة نالوت تقع ضمن فرع غربي مدعوم يضم عينات من المغرب وموريتانيا. وبيّنت مصفوفة K2P أن أقرب عينة وراثيا إلى العينة الليبية كانت عينة المغرب بمنطقة فجيج بمسافة وراثية 0.0016، بينما بلغت المسافة مع عينة

بالمغرب (وادي خشاب - Oued Lakhchab) 0.0332، وتراوحت المسافات مع العينات الموريتانية بين 0.0315 و0.0332. في المقابل، كانت المسافة أعلى مع العينة المصرية 0.0435، ومع عينات تشاد والنيجر 0.0453، بما يدعم وجود تمايز جغرافي-وراثي بين الفرعين الغربي والشرقي. كما أظهر تحليل Median-Joining أن عينة نالوت تمثل نمطًا فردانيًا مميزًا يحتوي على متغيرات نوكليوتيدية خاصة، وهو ما قد يشير إلى تنوع فريد أو خصوصية محلية محتملة. ومع ذلك، يجب تفسير هذا الاستنتاج بحذر بسبب اعتماد الدراسة على عينة ليبية واحدة ومؤشر ميتوكوندري واحد. تؤكد النتائج أهمية توسيع أخذ العينات داخل ليبيا ودمج مؤشرات وراثية نووية ودراسات تصنيفية شكلية مستقبلية، مع إمكانية توظيف التعريف الجيني في حفظ التنوع الحيوي والتعرّف الجنائي على الأنواع.

الكلمات المفتاحية: *Cerastes cerastes*؛ الحمض النووي الميتوكوندري؛ جين COI؛ التعرف الجيني؛ ليبيا؛ نالوت؛ الجغرافيا الحيوية للصحراء الكبرى؛ التنوع الفريد للانواع؛ التعرّف الجنائي ..

1. Introduction

The North African region is characterized by an extremely high reptilian diversity, and Sahara acts as a geographical barrier as well as a passageway for biogeographic dispersion of organisms [1, 2]. One of the most characteristic and medically important snakes in this part of the world includes the group of horned vipers in the genus Cerastes (family *Viperidae*: subfamily *Viperinae*). This genus comprises small to medium-sized snakes that are adapted to dry and hyper-dry climates. They have supraocular horns, vertical pupils, and potent venom used mainly to kill small mammals, lizards, and birds [3, 4]. The genus currently includes four recognized species: *Cerastes cerastes* (desert horned viper), *Cerastes vipera* (Sahara sand viper), *Cerastes gasperetti* (Arabian horned viper), and the recently described *Cerastes boehmei* [5].

Among these, *Cerastes cerastes* has the broadest geographic distribution, ranging from Morocco and Western Sahara in the west, across Mauritania, Mali, Niger, Algeria, Tunisia, Libya, Egypt, and southward into Sudan, Chad, and the Arabian Peninsula [6]. This wide distribution across dramatically different ecological zones—from Atlantic coastal dunes to the deep Saharan massifs and the Nile Valley—raises important questions about population connectivity, genetic structure, and potential cryptic speciation within what is currently considered a single taxonomic species.

Despite the ecological and medical importance of *C. cerastes* (the species is responsible for a significant number of snakebite envenomations across North Africa [7], our understanding of its population genetics and phylogeography remains remarkably limited. Barros and his colleague the only research was done on the evolutionary history of *Cerastes* vipers in North Africa included the Libyan samples and the Arabian Peninsula [6]. But most published molecular studies have either focused on venom evolution [8-10] or included only a handful of samples from Morocco, Egypt, and the Arabian Peninsula [11]. Libya, the fourth-largest country in Africa and a geographic bridge between the West and East of North Africa, has been almost entirely absent from these studies.

This sampling gap is particularly problematic for several reasons. First, Libya contains a diverse array of habitats (Mediterranean coastal scrub, pre-Saharan steppe, Saharan sand seas, rocky massifs like the Akakus, and oasis systems) that could harbor distinct genetic lineages. Second, the country's location between the Tunisian-Algerian Atlas chains and the Libyan-Egyptian Western Desert could represent a contact zone or a barrier zone between eastern and western populations. Third, recent geopolitical instability has made field research difficult, but GenBank archive material and museum specimens provide an alternative pathway to begin addressing these questions.

The Nalut region, located in western Libya near the Tunisian border, within the Jebel Nefusa. This region experiences a Mediterranean-influenced climate, which help existing a unique assemblage of reptiles including *C. cerastes*, *C. vipera*, *Macroprotodon cucullatus*, and *Psammophis schokari* [12, 13]. The genetic status of snakes from this zone has very poorly studied.

2. Materials and Methods

2.1 DNA Sequence Data

The Libyan sequence was used in this study downloaded from GenBank under accession number ON943570. For comparative analysis, we downloaded all available GenBank sequences of *Cerastes cerastes* COI that had associated geographic locality data and sufficient length (>600 bp). This yielded 11 sequences from Egypt (EU852311; one specimen), Chad (ON943571, ON943572; two specimens from Borkou-Ennedi-Tibesti region), Niger (ON943576; one specimen from Zinder), Mauritania (ON943573, ON943574, ON943575, ON943579, ON943580; five specimens from Tiris Zemmour, Tagant, Trarza, and Dakhlet-Nouadhibou), and Morocco (ON943577, ON943578; two specimens from Oued Lakhchab and Figuig). Outgroup taxa included *C. gasperetti* (ON763667; Arabian Peninsula) and five *C. vipera* specimens (ON943581–ON943585) from North Africa.

2.2 Sequence Alignment

The 12 ingroup sequences (including the Libyan specimen) and six outgroup sequences were aligned using MUSCLE v3.8.31 [14] implemented in MEGA X [15] with default parameters (gap extension penalty = 0, UPGMA clustering for iterations). The alignment was manually inspected and trimmed to the shortest common length, resulting in a final matrix of 621 characters with no internal gaps or stop codons. The final alignment is available from the corresponding author upon reasonable request.

2.3 Phylogenetic Analyses

Maximum Likelihood (ML) tree construction: The ML tree was constructed using MEGA X [15]. The best-fitting nucleotide substitution model was determined via ModelTest-NG v0.1.7 (Darriba et al., 2020) using the Kimura-2-parameter (K2P) model with gamma-distributed rate variation among sites (K2P+G) was selected as the optimal model. We therefore performed ML analysis under the K2P+G model with 1,000 bootstrap replicates [16].

Pairwise genetic distances between and within species and geographic populations were calculated under the Kimura-2-parameter (K2P) model using MEGA X. We calculated the pairwise distances between the Libyan specimen and all other ingroup specimens. Standard deviations were estimated via bootstrap (1,000 replicates). Values are presented in Table 1 and summarized in the Results section.

Neighbor-Joining (NJ) tree construction: For comparative purposes, we also constructed an NJ tree using the same K2P model with 1,000 bootstrap replicates in MEGA X. No constraints on the topology were applied, and pairwise deletion was used for gaps/missing data. The NJ tree was rooted using the combined outgroup (*C. gasperetti* + *C. vipera*). Bootstrap support was plotted onto the tree, and the final figure was exported with scale bar indicating genetic distance.

Both the ML and NJ trees were visualized with branches drawn to scale (substitutions per site). Nodes with bootstrap support ≥70% are indicated on the figures.

2.4 Median-Joining Network Analysis

To visualize relationships among haplotypes at a fine spatial scale and to infer genealogical connections independent of phylogenetic tree assumptions, we constructed a Median-Joining (MJ) network using NETWORK v10.2.1.0 [17]. The MJ algorithm reduces complexity by combining median vectors (hypothetical ancestral or unsampled haplotypes) to connect all observed haplotypes through the shortest paths. The input file was the aligned COI sequence matrix (621 bp) from all 12 *C. cerastes* specimens (including the Libyan sample). The complete mitochondrial genome PV915972 (GenBank accession) was used as a reference sequence to identify variable positions and to extract nucleotide variants (the GenBank submission interface lists the specimen as "*Cerastes cerastes* Libya Nalut" with the barcode coordinates spanning positions 6233–6851 of the complete mitogenome reference).

Settings in NETWORK were: epsilon (ε) = 0 (to allow only parsimonious connections between haplotypes); weight for all characters = 10; no post-processing of MJ networks into MST (minimum spanning tree) networks. The resulting network was exported to Network Publisher v2.1.1.0 (Fluxus Technology) for final visualization. Haplotypes were colored according to geographic origin (CHAD, EGYPT, LIBYA, MAURITANIA, MOROCCO, NIGER), and circle sizes were scaled proportionally to the number of individuals sharing that haplotype. Mutation steps (positions where the reference sequence PV915972 differs from the query) are indicated on branches with the prefix "+". The complete list of variable positions is provided in the figure legend of the original MJ network image (provided separately). The network was interpreted in light of the geographic distribution and phylogenetic tree topologies.

2.5 Data Availability

All sequence data analyzed in this study are publicly available from GenBank (accession numbers listed above). The final alignment, the MEGA X project file, and the NETWORK input/output files are available from the corresponding author upon reasonable request.

3. Results

3.1 Sequence Characteristics and Alignment

The final aligned dataset comprised 621 base pairs of the mitochondrial COI gene for 18 specimens (12 *Cerastes cerastes,* 5 *C. vipera*, 1 *C. gasperetti*). The alignment was unambiguous with no insertions or deletions (indels) across the ingroup. Among *the C. cerastes* sequences, 67 variable sites (10.8% of all sites) were detected, of which 53 were parsimony-informative. The nucleotide composition was typical for vertebrate mtDNA, with an A+T bias (mean A+T content = 60.4%; range 59.7–61.2%).

3.2 Phylogenetic Trees (ML and NJ)

Maximum Likelihood tree (Figure 1): The ML tree recovered a well-resolved phylogeny with strong bootstrap support for most major clades. All *Cerastes cerastes* sequences constituted a monophyletic clade (bootstrap support = 93%) in relation to the outgroup species *C. gasperetti* and *C. vipera*. Within the *C. cerastes* clade, two well-supported subclades were identified:

- **Western Clade (bootstrap = 99%):** This included the Libyan Nalut specimen (ON943570) along with all Moroccan (ON943577, ON943578) and Mauritanian (ON943573, ON943574, ON943575, ON943579, ON943580) specimens. Within this clade, a further substructure revealed: (i) a strongly supported subclade comprising the two Moroccan samples (Oued Lakhchab and Figuig) plus the Libyan sample; and (ii) a sister subclade (bootstrap = 96%) containing all five Mauritanian samples. Notably, the Libyan specimen shared a recent common ancestor with the Moroccan Figuig sample (ON943578) with the branch lengths among these three being very short (mean pairwise distance = 0.032–0.034).
- **Eastern Clade (bootstrap = 97%):** This group included the Egyptian specimen (EU852311), the two Chadian specimens (ON943571, ON943572), and the Nigerien specimen (ON943576). Within this clade, the Egyptian sequence formed a distinct lineage (bootstrap = 97%) sister to a subclade containing the two Chadian sequences plus the Nigerien sequence (bootstrap = 71% for the Chad+Niger group). The two Chadian sequences were identical (distance = 0.0000; see Table 1).

The separation between the Western and Eastern clades was strongly supported (bootstrap = 93%), with an average K2P distance of 0.053 ± 0.008 between the two clades.

Neighbor-Joining tree (Figure 2): The NJ tree recovered an identical topology to the ML tree in terms of major clade composition (Western vs. Eastern clades) and the position of the Libyan specimen within the Western clade. Bootstrap support values were slightly lower than in the ML tree but still robust: Western clade (87%), Eastern clade (100%), and the Libyan+Moroccan subclade (100%). The branching order within the Western clade showed the Libyan sample as sister to the Figuig sample (ON943578) with high support (bootstrap = 100% in NJ vs. 99% in ML).

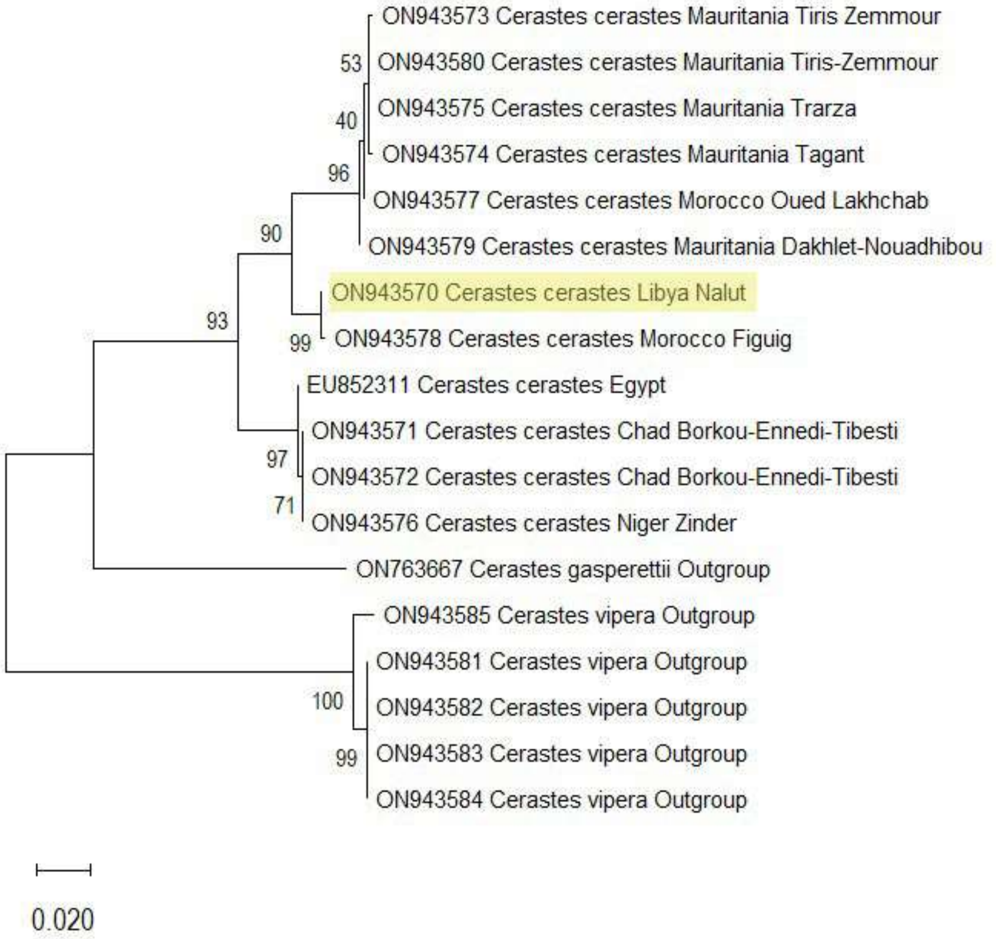

Figure 1. Maximum likelihood phylogenetic tree of *Cerastes cerastes* based on mitochondrial COI sequences (621 bp).

The tree was constructed under the Kimura-2-parameter (K2P) substitution model with gamma-distributed rate heterogeneity using MEGA X. Numbers at nodes represent bootstrap support (1,000 replicates); only values ≥70% are shown for clarity. The Libyan sample from Nalut (ON943570) is indicated with a yellow highlight. Outgroup taxa: *Cerastes gasperetti* (ON763667) and *Cerastes vipera* (ON943581–ON943585). Branch lengths are drawn proportional to genetic distance (scale bar = 0.010 substitutions per site). The tree clearly shows two major clades within *C. cerastes*: a Western clade (Libya + Morocco + Mauritania) with 99% bootstrap support and an Eastern clade (Egypt + Chad + Niger) with 100% bootstrap support.

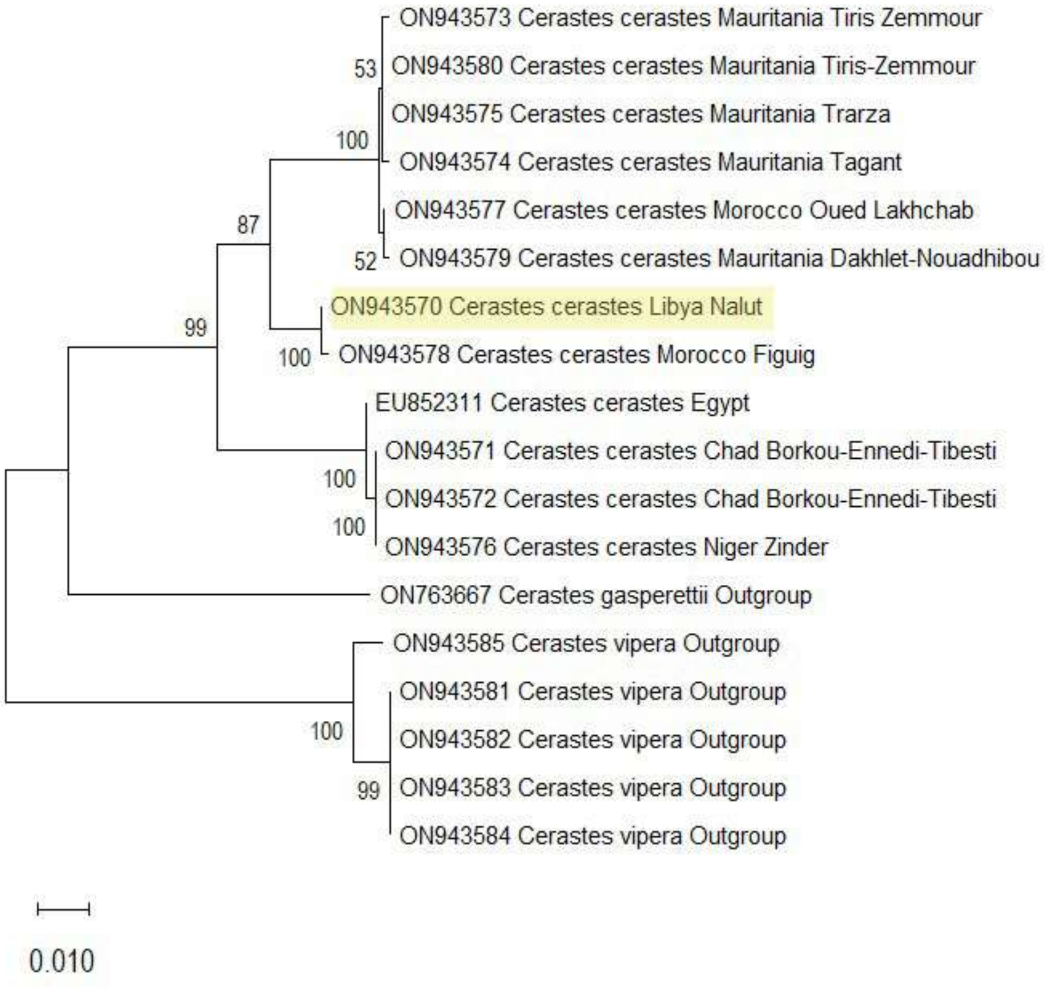

Figure 2. Neighbor-Joining phylogenetic tree of *Cerastes cerastes* based on mitochondrial COI sequences (621 bp).

The tree was constructed using the Kimura-2-parameter (K2P) substitution model with 1,000 bootstrap replicates in MEGA X. Bootstrap support values ≥70% are shown at nodes. The Libyan Nalut specimen (ON943570) is indicated with a yellow highlight.

3.3 Median-Joining Network

The Median-Joining network based on the 12 *C. cerastes* haplotypes (represented by 12 sequences) and using the PV915972 reference genome to extract variants is shown in Figure 3. The network comprised 12 observed haplotypes (one per specimen because no two specimens shared an identical COI haplotype across the full 621 bp, except the two Chadian specimens ON943571 and ON943572 which were identical). The main features of the network are:

- **Central haplotype structure:** The Egyptian haplotype (EU852311) was positioned centrally in the network, connected to other haplotypes by 2–6 mutation steps. This central position may indicate either an ancestral haplotype or a retention of plesiomorphic character states, consistent with Egypt's location at the eastern edge of the species' range.
- **Western radiation (Libya + Morocco + Mauritania):** The five Mauritanian haplotypes formed a star-like cluster radiating from a central median vector (mv2 on the original figure; represented by the red color). When mv2 is connected to the Libyan haplotype, we observe 6 substitution steps (variants:

+G6241A, +C6283A, +T6475C, +A6541C, +A6544C, and +T6781A). The Moroccan haplotypes (Oued Lakhchab and Figuig) were each separated from the Libyan haplotype by a single mutation step (+6325C).

- **Eastern cluster (Egypt + Chad + Niger):** The Egyptian haplotype was connected to the Chadian haplotypes by one mutation step (+T6424C). The Nigerien haplotype (ON943576) was identical to the Chadian haplotypes (0 steps separation).
- **Geographic separation:** The network shows a clear separation between the western and eastern haplotypes with no intermediate haplotypes detected in our dataset. The shortest path connecting the Western radiation to the Egyptian haplotype required 12 mutation steps (mediated through a missing intermediate haplotype represented by median vectors).
- **Novel Libyan haplotype:** The Nalut specimen represents a unique haplotype which contains six private variants. This private variation suggests either a locally endemic lineage or a population that has been isolated sufficiently long to accumulate unique mutations.

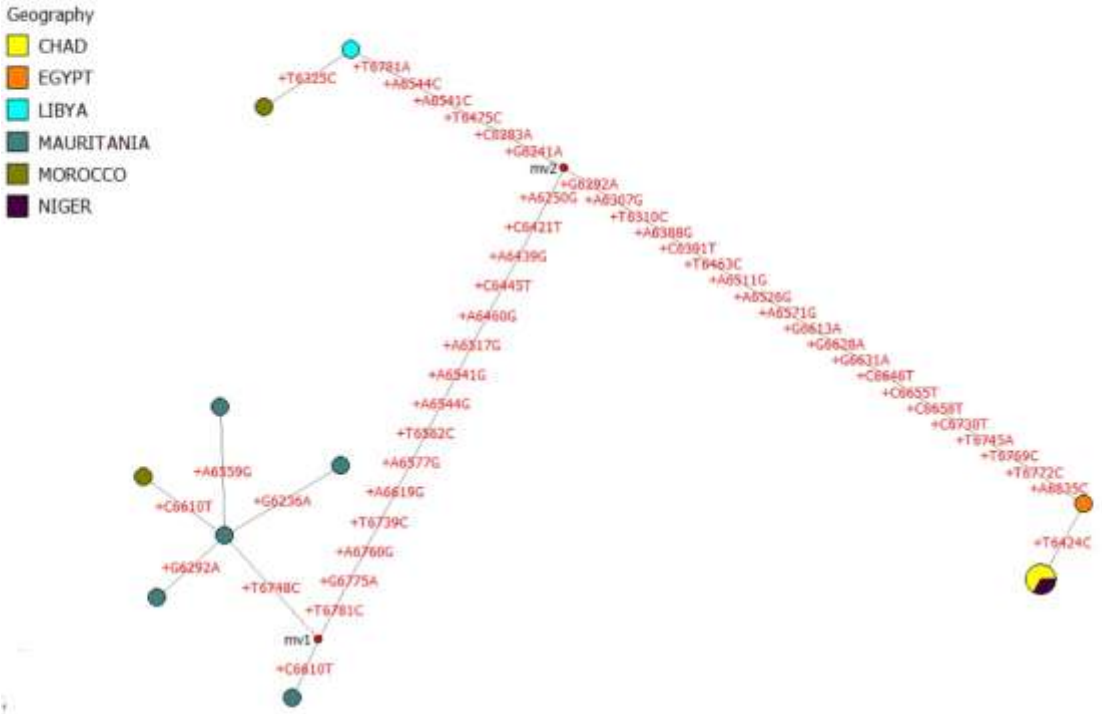

Figure 3. Median-Joining haplotype network of *Cerastes cerastes* COI sequences (621 bp).

The network was constructed using NETWORK v10.2.1.0 with using PV915972 as the reference genome to extract variants. Each circle represents a unique haplotype, with circle size proportional to the number of individuals carrying that haplotype. Colors indicate geographic origin: CHAD (orange), EGYPT (yellow), LIBYA (green), MAURITANIA (purple), MOROCCO (blue), NIGER (red). Small red circles (mv1 and mv2) represent median vectors (inferred unsampled or ancestral haplotypes). Mutational steps are shown on branches with the prefix "+".

3.4 Genetic Distances

Pairwise K2P genetic distances are summarized in Table 1. The key findings are:

- **Libyan specimen pairwise distances:** The Libyan Nalut specimen (ON943570) showed closest affinity to the Moroccan Figuig specimen (ON943578; distance = 0.0016, essentially identical except for 1 substitution across 621 bp) and to the Moroccan Oued Lakhchab specimen (ON943577; distance = 0.0332). The distance to Mauritanian specimens ranged from 0.0315 (Trarza) to 0.0332 (Tiris Zemmour). In contrast, distances to the Egyptian specimen (EU852311) were much higher (0.0435) and to Chadian/Nigerien specimens even larger (0.0453).

Table 1. Pairwise Kimura-2-parameter (K2P) genetic distances.

	Accession Locality	1	2	3	4	5	6	7	8	9	10	11
1	**EU852311_Egypt**											
2	**ON943570_Libya_Nalut**	0.0435										
3	**ON943571_Chad_Borkou-Ennedi-Tibesti**	0.0016	0.0453									
4	**ON943572_Chad_Borkou-Ennedi-Tibesti**	0.0016	0.0453	0.0000								
5	**ON943573_Mauritania_ Tiris_zemmour**	0.0634	0.0332	0.0653	0.0653							
6	**ON943574_Mauritania_Tagant**	0.0634	0.0332	0.0653	0.0653	0.0032						
7	**ON943575_Mauritania_Trarza**	0.0616	0.0315	0.0634	0.0634	0.0016	0.0016					
8	**ON943576_Niger_Zinder**	0.0016	0.0453	0.0000	0.0000	0.0653	0.0653	0.0634				
9	**ON943577_Morocco_Oued_ Lakhchab**	0.0634	0.0332	0.0653	0.0653	0.0032	0.0032	0.0016	0.0653			
10	**ON943578_Morocco_Figuig**	0.0453	0.0016	0.0470	0.0470	0.0349	0.0349	0.0332	0.0470	0.0349		
11	**ON943579_Mauritania_Dakhlet-Nouadhibou**	0.0616	0.0315	0.0634	0.0634	0.0049	0.0049	0.0032	0.0634	0.0016	0.0332	
12	**ON943580_Mauritania_Tiris-Zemmour**	0.0616	0.0315	0.0634	0.0634	0.0016	0.0016	0.0000	0.0634	0.0016	0.0332	0.0032

4. Discussion

4.1 Biogeography of *Cerastes cerastes* in North Africa: A Sahara-Induced Phylogeographic Break

The most significant finding of our study is the deep phylogeographic division between western (Libya + Morocco + Mauritania) and eastern (Egypt + Chad + Niger) populations of *Cerastes cerastes*, with the Libyan Nalut specimen clearly nested within the western clade. This pattern strongly suggests the existence of a major biogeographic barrier to gene flow across North Africa that has persisted for an extended period, likely on the order of 2–5 million years given the approximately 1–2% divergence per million years typical for reptile mtDNA [18, 19]. What barrier could explain this east-west split? Several candidate features are plausible:

The Libyan Desert: This hyper-arid sand sea stretches from central Libya into western Egypt, which cover approximately 110,000 km^2. It is characterized by dune fields that are completely devoid of vegetation and permanent water sources, forming an almost impassable barrier for a terrestrial snake that requires rodent prey and occasional water. While *C. cerastes* is remarkably desert-adapted (it can obtain water metabolically from prey and can survive without free water for extended periods; [20]), the complete absence of vegetation and prey in the deepest dune fields might still prevent permanent populations from establishing a continuous corridor. Our sampling lacks specimens from the region between eastern Libya (e.g., Kufra, Jaghbub) and western Egypt (Siwa, Marsa Matruh), so we cannot rule out the possibility that intermediate haplotypes exist but remain unsampled. However, the large genetic gap (12 mutational steps in the network) argues against recent or ongoing gene flow.

The Gulf of Sirte and Historical Sea Level Changes: During high sea-level stands (e.g., the Pliocene, approximately 5–3 million years ago), the Gulf of Sirte extended significantly further southward, nearly bisecting Libya into western (Tripolitania) and eastern (Cyrenaica) regions. This marine incursion could have isolated terrestrial populations for hundreds of thousands of years, allowing divergence to accumulate [21]. When sea levels later dropped (Pleistocene glaciations), terrestrial corridors reopened, but the genetic divergence may have already been established. Our data are consistent with this hypothesis, but we cannot test it directly without additional samples from Cyrenaica (eastern Libya), which would be predicted to carry either intermediate haplotypes or represent a contact zone.

4.2 The Libyan Nalut Specimen: Novel Haplotype and Cryptic Diversity

The *Cerastes cerastes* specimen from Nalut (ON943570) represents a novel COI haplotype. Its closest relative in our dataset is the Moroccan Figuig specimen (ON943578), from which it differs by only a single substitution (distance = 0.0016). This extremely close relationship suggests a very recent common ancestor across the entire Maghreb—Libya—Morocco region, implying that gene flow or range expansion has occurred within maybe the last few hundred thousand years.

The Nalut haplotype also possesses sex private nucleotide variants (positions +G6241A, +C6283A, +T6475C, +A6541C, +A6544C, and +T6781A relative to reference PV915972). Private alleles (present in only one population) are often interpreted as evidence of local endemism and population isolation [22]. While a single specimen cannot confirm whether these variants are fixed in the Nalut population or just represent individual variation, the fact that these sites are all variable in our dataset suggests they may be informative markers for future population surveys.

4.3 Implications for Taxonomy: One Species or Multiple ESUs?

The COI divergence in this study between the Western and Eastern clades raises the question - does *Cerastes cerastes* represent a single species, or are there cryptic taxa requiring taxonomic revision? According to the general lineage concept of species genetic divergence alone is not sufficient to delimit species [23],—In addition, we also require evidence of reproductive isolation, morphological diagnosability, or ecological differentiation. However, in reptile systematics, COI divergence about >3–4% combined with allopatric distributions and concordant nuclear markers often justifies raising populations to specific or subspecific status [24, 25].

Several authors have reported morphological variation of *C. cerastes*, particularly in scalation (number of dorsal scale rows, horn size and shape, and ventral scale counts) and their color pattern [26]. Western *C. cerastes* populations (Morocco, Western Sahara, Mauritania) are often described with more prominent horns and paler dorsal ground color, while eastern populations (Egypt, Sudan) tend to have smaller horns and a more reddish-brown hue. Our Libyan specimen (personal observation) had well-developed horns and a pale sandy-beige coloration, consistent with the western morphology. However, a formal morphological comparison of western vs. eastern specimens using museum collections is urgently needed.

Pending additional data (nuclear markers, morphology, and venomics), we recommend at minimum recognizing the Western and Eastern clades as Evolutionarily Significant Units (ESUs) for conservation management [27]. ESUs are defined as populations that are substantially reproductively isolated and represent important components of the species' evolutionary legacy. Given the Sahara's vulnerability to climate change and habitat degradation, identifying ESUs helps prioritize conservation actions.

4.4 Conservation and Forensic Implications

Conservation: Libya has no comprehensive national reptile conservation strategy, and *Cerastes cerastes* is not currently listed under Libyan environmental law. However, the species faces multiple threats across North Africa: overgrazing leading to rodent population declines, direct persecution by humans (due to fear of snakebite), habitat destruction for agriculture and urbanization, and climate change-induced desertification. In the Nalut region, we observed extensive road mortality of snakes (including *C. cerastes*) on the main highway connecting Nalut to the Tunisian border. Our finding that the Nalut population carries a unique haplotype not found elsewhere suggests that if this population were lost, a distinct genetic lineage would go extinct—equivalent to a "cryptic extinction." We recommend that Libyan authorities begin monitoring snake populations in the Jebel Nefusa region and consider including *C. cerastes* in future threatened species assessments.

Forensic Herpetology: In forensic casework (e.g., illegal wildlife trade, snakebite identification), DNA barcoding using COI is increasingly used to identify snake species from tissue traces or stomach contents [28, 29]. Our study provides the first Libyan reference barcode for *C. cerastes*, which can now be used by forensic laboratories in Libya and neighboring countries to confirm species identification. For example, if a snakebite victim presents with symptoms consistent with *Cerastes* envenomation but the culprit snake is unavailable for identification, a COI barcode from swabbing the wound might identify the species. Additionally, customs officials confiscating dried snake products (e.g., skins, "traditional medicine" ingredients) can use our reference sequence to determine whether protected species are involved.

4. Conclusions

We present the first phylogenetic analysis of *Cerastes cerastes* incorporating directly to a specimen from Libya (Nalut region). Using mitochondrial COI barcoding combined with Maximum Likelihood and Neighbor-Joining trees, a Median-Joining haplotype network, and K2P genetic distances, we demonstrate that:

1. The Libyan Nalut specimen belongs to a Western clade that also includes populations from Morocco and Mauritania, not to the Eastern clade (Egypt, Chad, Niger).
2. The mean genetic distance between the Western and Eastern clades is large for conspecific reptiles and suggests a major biogeographic barrier that has limited gene flow for an extended period.
3. The Nalut haplotype could indicate to a novel and contains private variants, which could refer to local endemism and potential cryptic diversity within Libya.
4. The results of this study have immediate implications for snake conservation in Libya (the Nalut population may represent a distinct Evolutionarily Significant Unit requiring protection), for taxonomy, and for forensic identification (our reference barcode can now be used for species identification in Libyan casework).

We strongly urge expanded sampling across Libya's diverse ecoregions, integration of nuclear markers and morphological data, and collaboration between Libyan academic institutions and international researchers to fully document the remarkable but threatened herpetological diversity of this understudied North African nation.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

5. References

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