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Molecular Phylogeny of *Walterinnesia aegyptia* (Reptilia, Elapidae) Isolated from Ha'il Province, Saudi Arabia

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Abstract

Walterinnesia aegyptia is one of the most venomous snakes belonging to the family Elapidae found in the Middle East and Africa. In addition to its ecological importance, it is accused of millions of deaths due to snakebites. Because molecular identification of snakes is crucial for the antivenom drug industry, mitochondrial genes are used to identify, characterize, and infer genetic diversity among different venomous snake species. Data of *Walterinnesia* collected from samples across Saudi Arabia were compared based on the mitochondrial 16S and 12S rRNA sequences with other Elapidae related taxa to assess the phylogenetic relationship. The phylogenetic analysis strongly supports the monophyly of the genus *Walterinnesia* based on two genes that represent different species of Elapidae. In addition, a close relationship between *Walterinnesia aegyptia* and *W. morgani* was found. Our molecular data showed that *W. morgani* from Riyadh, Saudi Arabia, is nearly genetically identical (D = 0) with *W. aegyptia* from Ha'il and Riyadh, Saudi Arabia, and Sinai, Egypt. Further study is required based on more material and detailed morphological and genetic analysis.

Keywords

Elapidae, MtDNA, Phylogeny, Saudi Arabia, *Walterinnesia aegyptia*

1. Introduction

The family Elapidae consists of about 389 species, including the cobras, mambas, and sea snakes. In Saudi Araba, two species *Walterinnesia* represent it, which include mainly two species *W. aegyptia* [1], *W. morgani* [2], and its relative spe-

cies, which belong to the *Naja* genus including one species reported in Saudi Arabia, the *N. haje arabica* [3]. The geographical distribution of the Arabian cobra (*N. haje arabica*) is the southwestern region while *W. aegyptia* belongs to the central, northern, and western regions of Saudi Arabia. However, *W. morgani* is distributed along with the Eastern parts of Saudi Arabia [4] [5] [6] [7] [8].

Snakebites represent a neglected health problem worldwide as well as in Saudi Arabia (https://www.who.int/news-room/fact-sheets/detail/snakebite-envenoming), [9] [10] [11]. In a recent review, 1688 snake bite cases were reported in Arab countries from 1983 to 2010 [12]. *W. aegyptia* envenomation causes respiratory failure and muscle paralysis in mice and humans, followed by rapid death when lethal doses of the venom are injected [12] [13] [14]. To the best of our knowledge, only two previous studies on phylogenetic analysis (using venom and/or 16S and 12S rRNA mitochondrial genes) of *Walterinnesia* and *Naja* snake species samples collected from across Saudi Arabia [15] [16]. Therefore, the present study aimed to determine the phylogenetic relationships in *Walterinnesia* and *Naja* species in Saudi Arabia using the mitochondrial 16S and 12S rRNA gene sequences.

2. Materials and Methods

2.1. Materials

Two samples of *W. aegyptia* were collected from Hail, Saudi Arabia, and identified morphologically according to Egan [17] and Alshammari and Ibrahim [6] (**Figure 1** and **Table 1**).

2.2. Methods

2.2.1. DNA Extraction, Amplification, and Sequencing

DNA was extracted using the Axy Prep Blood Genomic DNA Miniprep kit (Axygen Biosciences, USA), amplified, and sequenced for the 12S and 16S rRNA genes as described by Alshammari [18]. The obtained sequences were analyzed and submitted to GenBank (**Table 1**). Additional sequences of *W. aegyptia* from Saudi Arabia and Egypt, as well as available data sequences for other species of genus *Walterinnesia* from Saudi Arabia (**Table 1**) were downloaded from GenBank. Additional sequences of other genera were retrieved from GenBank to investigate the phylogenetic relationships of *Walterinnesia* within Elapidae. *Thermophis baileyi* [19] was used as an outgroup.

2.2.2. Phylogenetic Analyses

Finch TV 1.4.0 was used to screen and analyze the sequences, which were aligned using Clustal W [20] in Mega 6 using the default settings [21]. The aligned 12S and 16S sequences were concatenated and combined into a single alignment using the Mesquite v3.2 software [22], and the nucleotide composition was calculated. To estimate the sequence divergence for the whole data set, genetic distances were calculated using Mega v.6. Phylogenetic analyses were

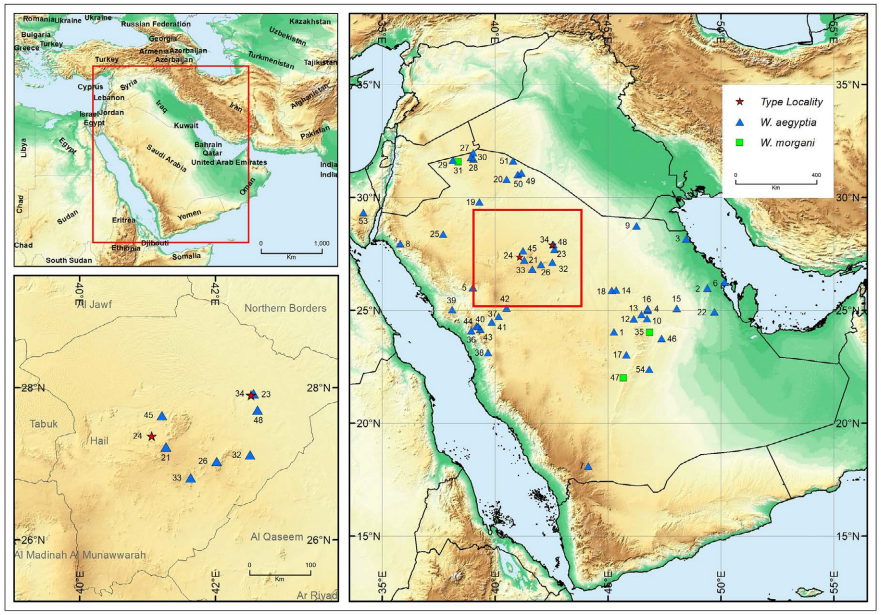

Figure 1. Records and collection records of *Walterinnesia sp.* in Saudi Arabia and North Africa. Sources of records are given in Table 1.

Table 1. Records of *Walterinnesia sp.* reported over the period from 1930 to 2022 and their accession number submitted to GenBank (from Saudi Arabia) (HUM = Ha'il University Museum).

No.	Species	Location	Coordinates	12S	16S	MC1R	References
1	***W. aegyptia***	Al-Quwayiah	24˚03'N 45˚15'E	-	-	-	
2	***W. aegyptia***	Ayan Dar	25˚59'N 49˚23'E	-	-	-	
3	***W. aegyptia***	AlMishab	28˚12'N 48˚37'E	-	-	-	
4	***W. aegyptia***	30 mi. N of Riyadh	25˚04'N 46˚45'E	-	-	-	
5	***W. aegyptia***	NW of Khaybar	26˚N 39˚'E	-	-	-	
6	***W. aegyptia***	Thuqbah	26˚15'N 50˚09'E	-	-	-	
7	***W. aegyptia***	Wadi Qatan	18˚06'N 44˚07'E	-		-	[38]
8	***W. aegyptia***	Jabal as Sinfa	27˚57'N 35˚47'E	-	-	-	
9	***W. aegyptia***	NE of Hafr AlBatin	28 3/4˚N 46 1/4˚E	-	-	-	
10	***W. aegyptia***	N of Riyadh	25˚N 46 3/4˚E	-	-	-	
11	***W. aegyptia***	Durma	24˚37'N 46˚08'E	-	-	-	
12	***W. aegyptia***	Wadi Amariyyah	24˚49'N 46˚28'E	-	-	-	
13	***W. aegyptia***	Al Majmaah	25˚54'N 45˚21'E	-	-	-	

Continued

14	***W. aegyptia***	Khurays	25˚05'N 48˚02'E	-	-	-	
15	***W. aegyptia***	Ar Riyadh	24˚38'N 46˚43'E	-	-	-	[38]
16	***W. aegyptia***	Al Huwah	23˚02'N 45˚48'E	-	-	-	
17	***W. aegyptia***	Ar Ruwaydah	25˚53'N 45˚09'E	-	-	-	
18	***W. aegyptia***	Al-Jouf-South of Abo Ajram	29˚47'56"N 39˚17'16"E	-	-	-	[5]
19	***W. aegyptia***	Al-Jouf-Wadi Al-Aqra'a	30˚47'59"N 40˚30'16"E	-	-	-	
20	***W. aegyptia***	Ha'il-Aja Mountain	27˚13'35"N 41˚16'40"E	-	-	-	[39]
21	***W. aegyptia***	Al-Hasa	-	-	-	-	[40]
22	***W. aegyptia***	Ha'il, Baqa'a	27˚54'02.5"N 42˚31'56.9"E	HQ658416.1	HQ267785	-	This Study
23	***W. aegyptia***	Ha'il, Faid	27˚22'01.1"N 41˚04'07.7"E	-	-	-	
24	***W. aegyptia***	Tabouk-Alqelebah	28˚22'48"N 37˚41'39"E	-	-	-	[41]
25	***W. aegyptia***	Ha'il between Taba and Assaba'an	27˚02'15"N 42˚01'25"E	-	-	-	[6]
26	***W. aegyptia***	Turaif	31˚58'458"N 39˚01'918"E	-	-	-	
27	***W. aegyptia***	Turaif	31˚42'954"N 39˚02'424"E	-		-	
28	***W. aegyptia***	Turaif	31˚58'603"N 38˚02'102"E	-	-	-	[5]
29	***W. aegyptia***	Turaif	31˚46'231"N 38˚55'925" E	-	-	-	
30	***W. morgani***	Turaif	31˚58'603" N 38˚02'102"E	-	-	-	
31	***W. aegyptia***	Ha'il, Faid	27˚07'21"N 42˚31'12"E	-	-	-	
32	***W. aegyptia***	Ha'il, Ar-Rawdha	26˚49'18"N 41˚38'26"E	-	-	-	[8]
33	***W. aegyptia***	Ha'il, Baqa'a	27˚55'19"N 42˚34'09"E	-	-	-	
34	***W. morgani***	Riyadh	-	-	MW198209.1	-	[16]
35	***W. aegyptia***	Alab	24˚06'13.08"N 38˚55'48.13"E	-	-	-	
36	***W. aegyptia***	Suwaydrah	24˚43'31.77"N 40˚08'32.70"E	-	-	-	[42]
37	***W. aegyptia***	Wadi khadhrah	23˚07'26.71"N 39˚40'24.28"E	-	-	-	
38	***W. aegyptia***	Al Ays	25˚01'49.91"N 38˚05'21.45"E	-	-	-	

Continued

39	***W. aegyptia***	Hazarah	24˚17'23.53"N 39˚14'35.68"E	-	-	-	
40	***W. aegyptia***	Al Nekhael	25˚05'55.92"N 40˚29'34.05"E	-	-	-	
41	***W. aegyptia***	Wadi Alkhung	24˚28'41.64"N 39˚50'10.77"E	-	-	-	[42]
42	***W. aegyptia***	Wadi Reem	24˚10'13.58"N 39˚19'50.03"E	-	-	-	
43	***W. aegyptia***	Far'a Alradadi	24˚18'04.20"N 39˚10'08.18"E	-	--	-	
44	***W. aegyptia***	Ha'il, Hofair	27˚38'18.6"N 41˚13'13.9"E	-	-	-	Alshammari and Aloufi 2021 (HUM)
45	***W. aegyptia***	Riyadh	-	-	MZ520322.1	-	
46	***W. aegyptia***	Egypt Sinai	-	-	MZ520323	MZ520319.1	[43]
47	***W. aegyptia***	Riyadh	-	-	-	MZ520318.1	
48	***W. morgani***	Riyadh	-	MW198201.1	-	-	[16]
49	***W. aegyptia***	Ha'il, Jetheathah	27˚42'33.8"N 42˚37'36.5"E	-	-	-	Alshammari and Aloufi 2022 (HUM)
50	***W. aegyptia***	North Border-Ar'ar	31˚04'57"N 41˚09'50"E	-	-	-	
51	***W. aegyptia***	North Border-Ar'ar	31˚00'40"N 40˚59'09"E	-	-	-	[44]
52	***W. aegyptia***	North Border-Ar'ar	31˚37'25"N 40˚46'47"E	-	-	-	
53	***W. aegyptia***	-	-	U96807.1	-		[45]

performed on the combined data set (n = 19), as well as separate analyses on the individual gene was performed to determine the signal in the individual gene. The maximum parsimony (MP) and neighbor-joining (NJ) analyses were performed with Paup v.4 [23] with heuristic searches using stepwise addition followed by tree bisection reconnection (TBR) branch swapping [24]. In all alignments, gaps were treated as missing characters. Confidence within the nodes was evaluated using 1000 bootstrap replicates [25] [26] with random addition of taxa. MrModeltest 2.3 [27] was used to select the best-fit models of nucleotide evolution supported by Akaike information criterion (AIC) [28]. The geographic structure was inferred using Bayesian inference (BI) implemented with MrBayes 3.1.2 [29]. Analyses were run for one million generations and the output parameters were visualized to determine stationarity and convergence using Tracer 1.4 [30].

3. Results

Across all combined sequences, there were 799 aligned nucleotides. Of these, 655

bases (80.9%) were constant; 144 (18.0%) were variable, and 89 (12.2%) were parsimony informative. Within the 799 bp, 141 polymorphic segregating sites were detected. Divergence among *Walterinnesia and different* Elapidae related taxa ranged from 0 to 0.06 (**Table 2**). For the 16S rRNA fragment, there were 468 aligned sites, of which 323 (69.0%) bases were constant, 142 (30.3%) bases were variable, and 111 (23.7%) were parsimony informative. The mean values of T, C, A, and G within the sequence data were 23.6%, 23.6%, 34.1%, and 18.7%, respectively. Within the 468 bp, 31 polymorphic segregating sites were detected. The sequence divergences among *Walterinnesia* and Elapidae related taxa lineages ranged from 0.05 to 0.05 as represented in **Table 2**. For the 12S rRNA fragment, there were 390 aligned sites, of which 258 (66.1%) bases were constant, 123 (31.5%) bases were variable, and 97 (24.8%) were parsimony informative. Within the 413 bp, 30 polymorphic segregating sites were detected. The mean values of T, C, A, and G within the sequence data were 19.8%, 25.0%, 36.9%, and 18.3%, respectively. Within the 390 bp, 26 polymorphic segregating sites were detected. The sequence divergences among *Walterinnesia* and *Naja sp.* lineages ranged from 0.00 to 0.07 as shown in **Figure 2-4** and **Table 2**. The tree topologies were improved based on the 12 S rRNA and 16 S rRNA data sets. The NJ and MP analyses were comparable in defining two main clades. The first clade includes all taxa that belonged to the family Colubridae. Another clade encompassed all taxa belonging to the family Elapidae. In addition, the phylogenetic analysis revealed the monophyletic status of the genus *Walterinnesia* based on the 12s rRNA gene and the paraphyletic status between the genus *Walterinnesia* and *Naja*.

4. Discussion

The Saudi Arabia landscape has an extremely diverse topographic and bioclimatic profile resulting in the development of numerous habitats of diverse fauna and flora that is a blend of Indomalayan, Palearctic, and Ethiopian species [31] [32].

W. aegyptia, is known as the desert black snake or desert cobra. This species' geographical distribution includes northern and southern Saudi Arabia, southern Israel, western Jordan, and possibly Lebanon [5] [33]. Our data showed a generally well-supported phylogenetic hypothesis for the *Walterinnesia* and different Elapidae snake species across Saudi Arabia. This provides the raw material for a discussion of the evolution of spitting in cobras and long-standing taxonomic issues. Phylogenetic analysis using 16 S and 12 S mtDNA gene sequences has produced distinctive clades for *Walterinnesia* and *Naja* snake species.

The phylogenetic analyses (NJ, MP and PI) weakly support the monophyly of the genus *Walterinnesia*, based on two combined genes representing different species of Elapidae taxa (**Figures 2-4**). When analyzed separately, the 12S rRNA and 16s rRNA genes support the monophyly of *Walterinnesia*, *Naja*, and *Aspidelaps*. However, the 16S rRNA gene showed a sister relationship between *Walterinnesia* and *Aspidelaps* (Supplemental material **Figure 1**, **Figure 2**). Wüster

Table 2. Uncorrected pairwise distances among *Walterinnesia* samples based on concatenated mitochondrial 12S rRNA and 16S rRNA sequences. Standard error estimates are shown above the diagonal. SA = Saudi Arabia.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1) *Walterinnesia_aegyptia SA*																		
2) *Walterinnesia_morgani SA*	0.00																	
3) *Naja_haje*	0.05	0.05																
4) *Naja_kaouthia*	0.04	0.05	0.04															
5) *Naja_sumatrana*	0.05	0.05	0.05	0.02														
6) *Naja_annulifera*	0.05	0.05	0.01	0.04	0.05													
7) *Naja_haje_arabica*	0.05	0.05	0.00	0.04	0.05	0.01												
8) *Naja_pallida*	0.05	0.05	0.03	0.04	0.04	0.03	0.03											
9) *Naja_melanoleuca*	0.05	0.05	0.03	0.04	0.04	0.03	0.03	0.03										
10) *Naja_siamensis*	0.05	0.06	0.05	0.03	0.02	0.05	0.05	0.05	0.04									
11) *Naja_mossambica*	0.05	0.05	0.04	0.04	0.05	0.04	0.04	0.04	0.03	0.05								
12) *Sinomicrurus_kelloggi*	0.06	0.06	0.07	0.06	0.06	0.07	0.07	0.06	0.06	0.07	0.06							
13) *Naja_katiensis*	0.05	0.05	0.03	0.04	0.04	0.04	0.04	0.03	0.03	0.05	0.02	0.06						
14) *Naja_ashei*	0.05	0.05	0.04	0.05	0.05	0.04	0.04	0.04	0.03	0.05	0.01	0.07	0.02					
15) *Boulengerina_annulata*	0.06	0.06	0.06	0.06	0.06	0.05	0.06	0.05	0.05	0.07	0.05	0.06	0.05	0.05				
16) *Naja_atra*	0.05	0.05	0.05	0.01	0.02	0.05	0.05	0.04	0.03	0.02	0.05	0.06	0.04	0.05	0.06			
17) *Ophiophagus_hannah*	0.06	0.06	0.05	0.06	0.06	0.06	0.05	0.05	0.05	0.07	0.06	0.06	0.06	0.06	0.06	0.06		
18) *Sinomicrurus_peinani*	0.06	0.07	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.04	0.06	0.07	0.07	0.06	0.07	
19) *Thermophis_baileyi (Outgroup)*	0.07	0.08	0.08	0.07	0.08	0.07	0.08	0.07	0.07	0.08	0.08	0.08	0.08	0.08	0.08	0.07	0.08	0.08

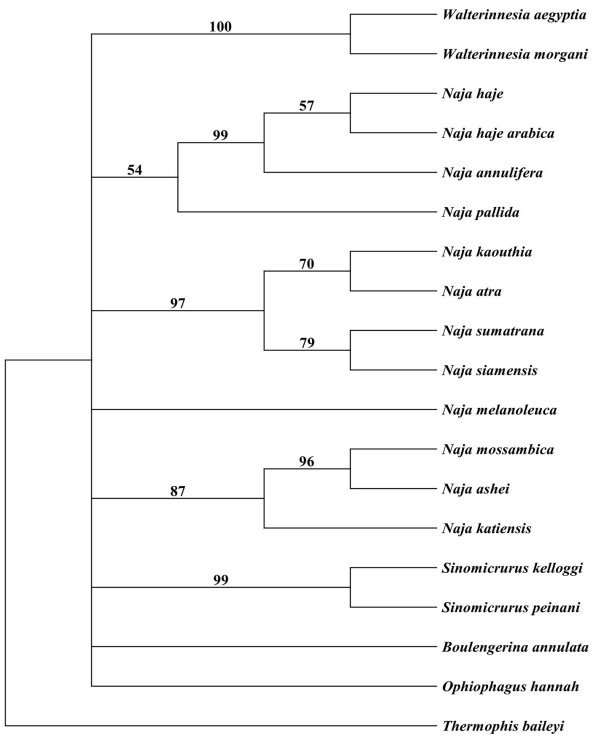

Figure 2. Maximum parsimony phylogenetic tree of the *Walterinnesia* species and Elapidae related taxa based on the concatenated mitochondrial 12S rRNA and 16S rRNA sequences. The number above the branches indicates bootstrap values.

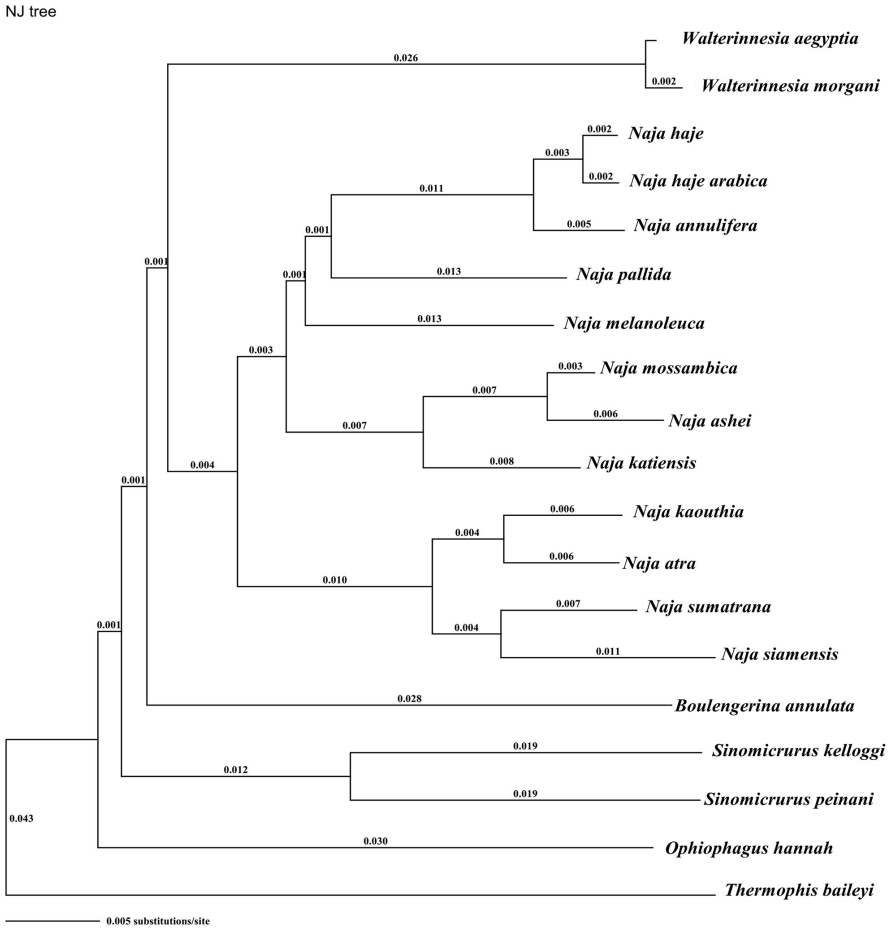

Figure 3. Neighbor-Joining phylogenetic tree of the *Walterinnesia* species and Elapidae related taxa based on the concatenated mitochondrial 12S rRNA and 16S rRNA sequences. The number above the branches indicates distance values.

et al. [34] confirmed the strongly supported monophyletic relationship between the core cobra clade, which includes *Aspidelaps*, *Boulengerina*, *Hemachatus*, *Naja*, *Paranaja*, and *Walterinnesia*. In addition, the two genera *Walterinnesia* and *Aspidelaps* formed two basal lineages to the other members of the cobra clade. However, the sister–group affinities between the above genuses remained uncertain [34] [35] [36] [37]. Our data strongly support the sister relationship between *Walterinnesia* and *Aspidelaps* based on the 16S and 12S rRNA genes.

Within the *Walterinnesia*, there is a strong support for the sister relationship between *W. aegyptia* and *W. morgani* based on 16S rRNA and 12S rRNA genes. The two species are closely similar and are related in their external morphology. *W. morgani* can be distinguished from *W. aegyptia* by its lower number of subcaudal and ventral scales in both sexes; a lower number of united subcaudals in females, by having a juvenile pattern of reddish crossbars on the back, and lower average ventral and subcaudal scale counts [4]. This information was originally described as *W. aegyptia* [1] from Egypt, or as *N. morgani* [2] or A*tractaspis wilsoni* [19] from Iran and they have appeared in the literature over time. Subsequently, the eastern populations represent a different species, *W. morgani* [4].

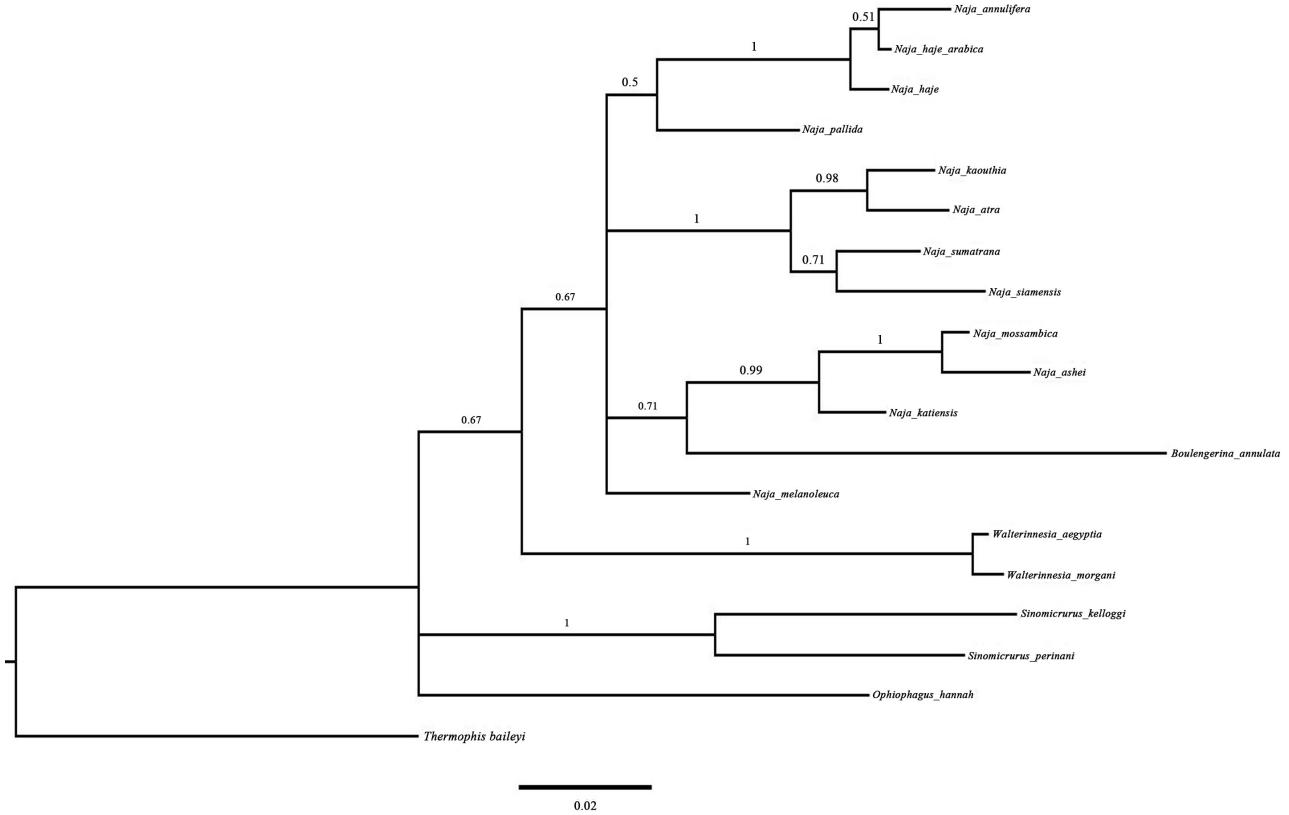

Figure 4. Bayesian inference tree of the *Walterinnesia* species and Elapidae related taxa based on the concatenated mitochondrial 12S rRNA and 16S rRNA sequences. The number above the branches indicates bootstrap values.

5. Conclusion

Our molecular data showed that *W. morgani* from Riyadh, Saudi Arabia, is nearly genetically identical (D = 0) to *W. aegyptia* from Ha'il and Ryiadh, Saudi Arabia, and Sinai, Egypt. As a result, it is certain that the samples identified as *W. morgani* from Riyadh, Saudi Arabia [16] are conspecific with *W. aegyptia*. Additional research based on more data and thorough morphological and genetic analysis is needed.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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