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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 congeners 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 a hundred species, including the cobras, mambas, and sea snakes. In Saudi Araba, two genera *Walterinnesia* represent it, which include mainly two species *W. aegyptia* (Lataste 1887), *W. morgani* (Mocquard 1905), and its relative species, which belong to the *Naja* genus including one species reported in Saudi Arabia, the *N. haje arabica* (Scortecci 1932). 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 (Nilson and Rastegar-Pouyani 2007; Al-Sadoon et al. 2017; Alshammari and Ibrahim 2015; Alshammari et al. 2017; Alshammari and Busais 2020).

Snakebites represent a neglected health problem worldwide and in Saudi Arabia https://www.who.int/news-room/fact-sheets/detail/snakebite-envenoming; Al-Sadoon *et al.* 2020). Several reviews reported 1,688 snake bites cases (from 1983 to 2010) and 20 deaths collectively (Amer et al. 2020). *W. aegyptia,* envenoming induces respiratory failure and muscle paralysis in mice and humans similar to other elapid venoms. Injecting lethal quantities of its venom into mice or rats resulted in quick death (Amr and Disi 2011; Sindaco *et al.* 2013; Amr et al. 2020). To the best of our knowledge, there is no previous study on phylogenetic analysis of *Walterinnesia* and *Naja* snake species samples collected across Saudi Arabia. Therefore, the present study was conducted to determine the phylogenetic relationship in *Walterinnesia* and *Naja* species collected from across Saudi Arabia using the mitochondrial 16S and 12S rRNA gene sequences.

Materials and Methods

Two samples of *W. aegyptia* were collected from Hail, Saudi Arabia, and identified morphologically according to Egan (2007) and Alshammari and Ibrahim (2015; Fig. 1 and Table 1). The DNA was extracted, amplified, and sequenced for the 12S and 16S rRNA genes as Alshammari (2011) described. 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 relationship between *Walterinnesia* within Elapidae. *Thermophis baileyi* (Wall, 1907) was used as an outgroup (MK194019.1 and MK065451.1).

Phylogenetic analyses

Finch TV 1.4.0 was used to screen and analyze the sequences, which were aligned using ClustalW in Mega 6 using the default settings (Tamura et al. 2013). To estimate the sequence divergence for the whole data set, genetic distances were calculated using Mega 6. Phylogenetic analyses were performed on separate analyses on the individual gene were performed to determine the signal in the individual gene. The maximum parsimony (MP) and neighbor-joining (NJ) analyses were performed with Paup v4 (Swofford 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 1,000 bootstrap replicates (Felsenstein 2002) with random addition of taxa.

Results and Discussion

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 *Naja sp.* lineages ranged from 0.05 to 0.05, which are represented in Table S1. 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 Figs. 2–5 and Table S2.

The tree topologies were improved based on the 12S rRNA and 16S 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. Finally, the paraphyletic status between the genus *Walterinnesia* and *Naja*.

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 (Addas et al. 2018; Sobaihi 2019). Of these, *W. aegyptia*, which 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 (Al-Sadoon et al. 2017; Aloufi et al. 2019). The present study was conducted to determine the evolutionary relationship. Our data showed a generally well-supported phylogenetic hypothesis for the *Walterinnesia* and *Naja* snake species collected from samples 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 16S and 12S mtDNA gene sequences produces distinctive clades for *Walterinnesia* and *Naja* snake species.

The phylogenetic analyses (NJ and MP) strongly support the monophyly of the genus *Walterinnesia*, based on two genes representing different species of Elapidae (Figs. 2–5). The 12S rRNA and 16s rRNA gene support the monophyletic of *Walterinnesia*, *Naja*, and *Aspidelaps*. However, the16S rRNA gene showed a sister relationship between *Walterinnesia* and *Aspidelaps.* Wüster et al. (2007) confirmed the strongly supported monophyly 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 genus remained uncertain (Zaher et al. 2019). 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 16SrRNA 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 subcaudals and ventrals 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 (Nilson and Rastegar-Pouyani 2007). This information was originally described as *W. aegyptia* (Lataste 1881) from Egypt, or as *Naja morgani* (Mocquard, 1905) or A*tractaspis wilsoni* (Wall 1908) from Iran and they have appeared in the literature over time. Subsequently, the eastern populations represent a different species, *Walterinnesia morgani* (Nilson and Rastegar-Pouyani 2007). However, our molecular data showed that *W. morgani* from Ryiadh, Saudi Arabia, is nearly genetically identical (D=0) with *W. aegyptia* from Ha`il and Ryiadh, Saudi Arabia, and Sinai, Egypt. Thus, the samples referred to *W. morgani* from Ryiadh, Saudi Arabia (Šmíd et al. 2021) are undoubtedly conspecific with *W. aegyptia*. Therefore, further study is required based on more material and detailed morphological and genetic analysis.

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

Figure 2. Maximum parsimony phylogenetic tree of genus *Walterinnesia* mtDNA sequences fragment of the 16S rRNA gene from Saudi Arabia. The number above the branches indicates bootstrap values calculated with 1,000 replicates.

Figure 3. Neighbor-joining phylogenetic tree of genus *Walterinnesia* mtDNA sequences fragment of the 16S rRNA gene from Saudi Arabia and Egypt including Sinai, Oman, and Turkey. The number above the branches indicates distance values.

Figure 4. Maximum parsimony phylogenetic tree of genus *Walterinnesia* mtDNA sequences fragment of the 12S rRNA gene from Saudi Arabia. The number above the branches indicates bootstrap values calculated with 1,000 replicates.

Figure 5. Neighbor-joining phylogenetic tree of genus *Walterinnesia* mtDNA sequences fragment of the 12S rRNA gene from Saudi Arabia and Egypt including Sinai, Oman, and Turkey. The number above the branches indicates distance values.

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	-	-	-	Gasperetti 1988
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	-		-	
8	***W. aegyptia***	Jabal as Sinfa	27° 57' N 35° 47' E	-	-	-	
9	***W. aegyptia***	NE of Hafr AlBatin	28 ¾° N 46 ¼° E	-	-	-	
10	***W. aegyptia***	N of Riyadh	25° N 46 ¾° 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	-	-	-	
14	***W. aegyptia***	Khurays	25° 05' N 48° 02' E	-	-	-	
15	***W. aegyptia***	Ar Riyadh	24° 38' N 46° 43' E	-	-	-	
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	-	-	-	Alsadoo et al. 2017
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	-	-	-	Sharawy and Alshammari 2009
21	***W. aegyptia***	Al-Hasa	-	-	-	-	Alsadoon 2010
22	***W. aegyptia***	Ha'il, Baqa'a	27° 54' 02.5" N 42° 31' 56.9" E	HQ658416.1	-	-	This paper
23	***W. aegyptia***	Ha'il, Faid	27° 22' 01.1" N 41° 04' 07.7" E	-	HQ267785	-	
24	***W. aegyptia***	Tabouk-Alqelebah	28° 22' 48" N 37° 41' 39" E	-	-	-	Aloufi and Amr 2015
25	***W. aegyptia***	Ha'il between Taba and Assaba'an	27° 02' 15" N 42° 01' 25" E	-	-	-	Alshammari and Ibrahim 2015
26	***W. aegyptia***	Turaif	31° 58' 458" N 39° 01' 918" E	-	-	-	Al-Sadoon et al. 2017
27	***W. aegyptia***	Turaif	31° 42' 954" N 39° 02' 424" E	-		-	
28	***W. aegyptia***	Turaif	31° 58' 603" N 38° 02' 102" E	-	-	-	

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	-	-	-	Alshammari and Busais 2020
32	***W. aegyptia***	Ha'il, Ar-Rawdha	26° 49' 18" N 41° 38' 26" E	-	-	-	
33	***W. aegyptia***	Ha'il, Baqa'a	27° 55' 19" N 42° 34' 09" E	-	-	-	
34	***W. morgani***	Riyadh	-	-	MW198209.1	-	Smid et. al. 2021
35	***W. aegyptia***	Alab	24° 06' 13.08" N 38° 55' 48.13" E	-	-	-	Aloufi et al. 2021
36	***W. aegyptia***	Suwaydrah	24° 43' 31.77" N 40° 08' 32.70" E	-	-	-	
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	-	-	-	
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	***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	-	Calvete et al. 2021
46	***W. aegyptia***	Egypt Sinai	-	-	MZ520323	MZ520319.1	
47	***W. aegyptia***	Riyadh	-	-	-	MZ520318.1	
48	***W. morgani***	Riyadh	-	MW198201.1	-	-	Smid et. al., 2021
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	-	-	-	Alshammari 2022 (in press)
51	***W. aegyptia***	North Border-Ar'ar	31° 00' 40" N 40° 59' 09" E	-	-	-	
52	***W. aegyptia***	North Border-Ar'ar	31° 37' 25" N 40° 46' 47" E	-	-	-	
53	***W. aegyptia***	-	-	U96807.1	-		Slowinski et al. 1997

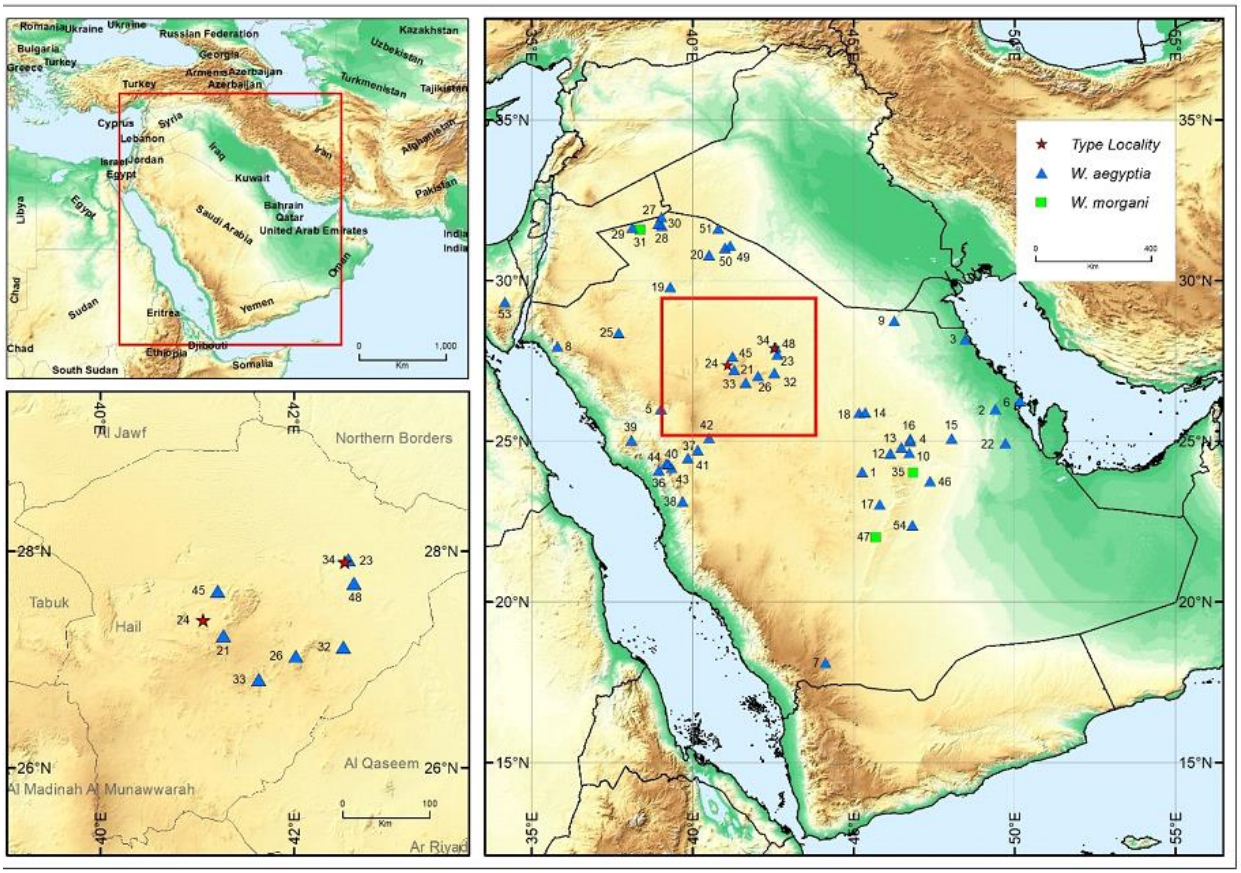

Figure 1. Records and collection records of *Walterinnesia sp.* in Saudi Arabia and North Africa. Sources of records are given in Table 1.

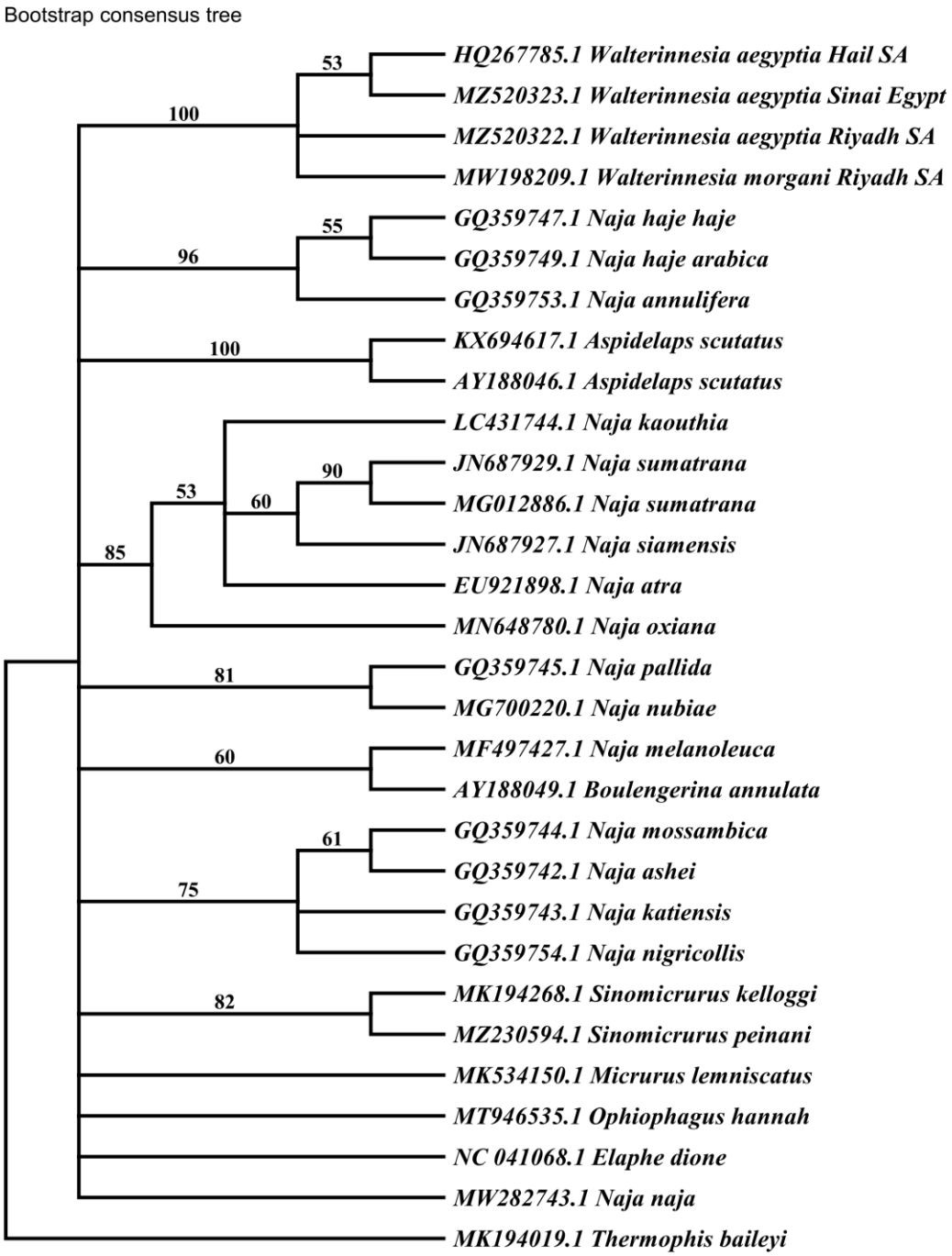

Figure 2. Maximum parsimony phylogenetic tree of genus *Walterinnesia* mtDNA sequences fragment of the 16S rRNA gene from Saudi Arabia. The number above the branches indicates bootstrap values calculated with 1,000 replicates.

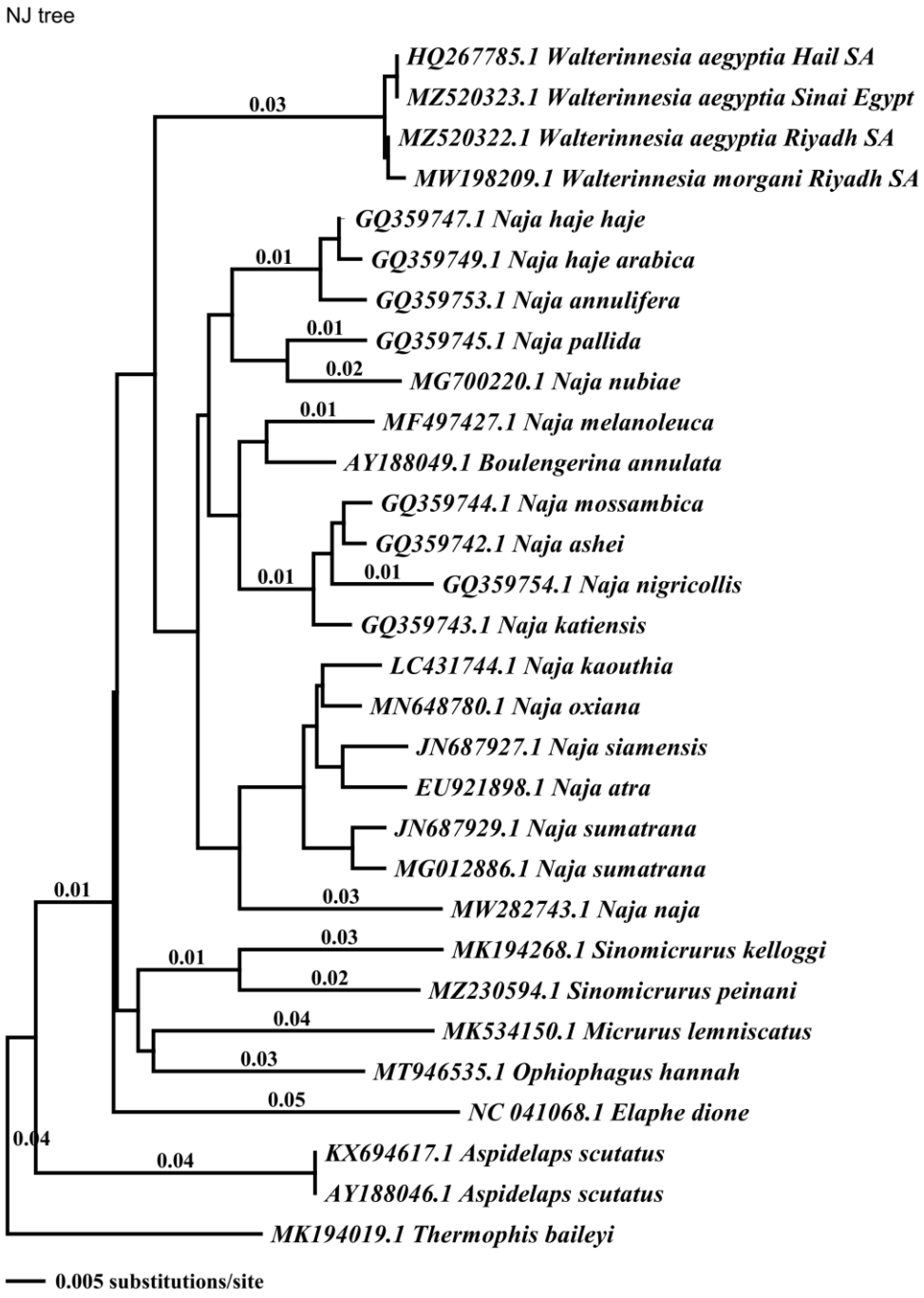

Figure 3. Neighbor-joining phylogenetic tree of genus *Walterinnesia* mtDNA sequences fragment of the 16S rRNA gene from Saudi Arabia and Egypt including Sinai, Oman, and Turkey. The number above the branches indicates distance values.

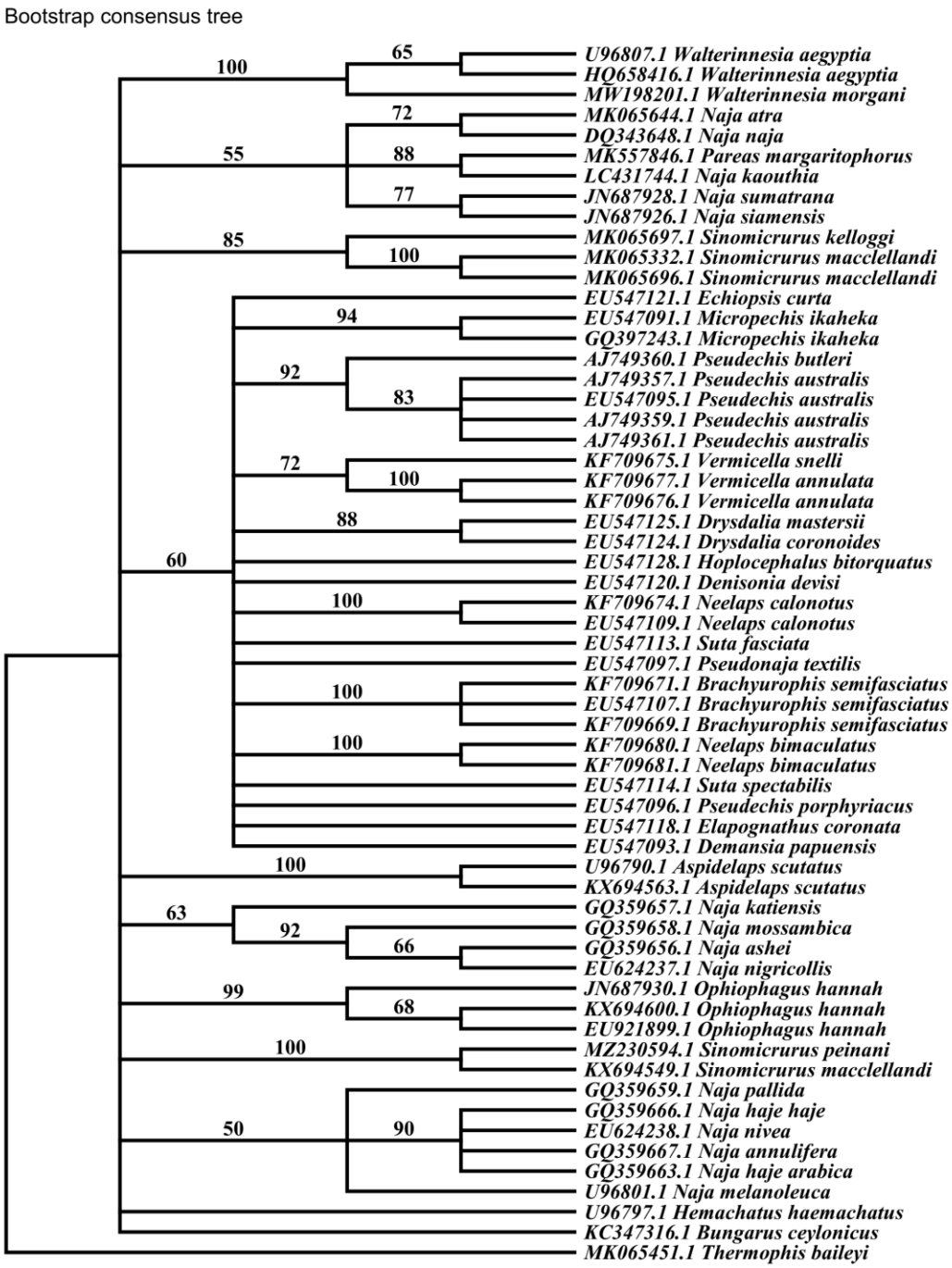

Figure 4. Maximum parsimony phylogenetic tree of genus *Walterinnesia* mtDNA sequences fragment of the 12S rRNA gene from Saudi Arabia. The number above the branches indicates bootstrap values calculated with 1,000 replicates.

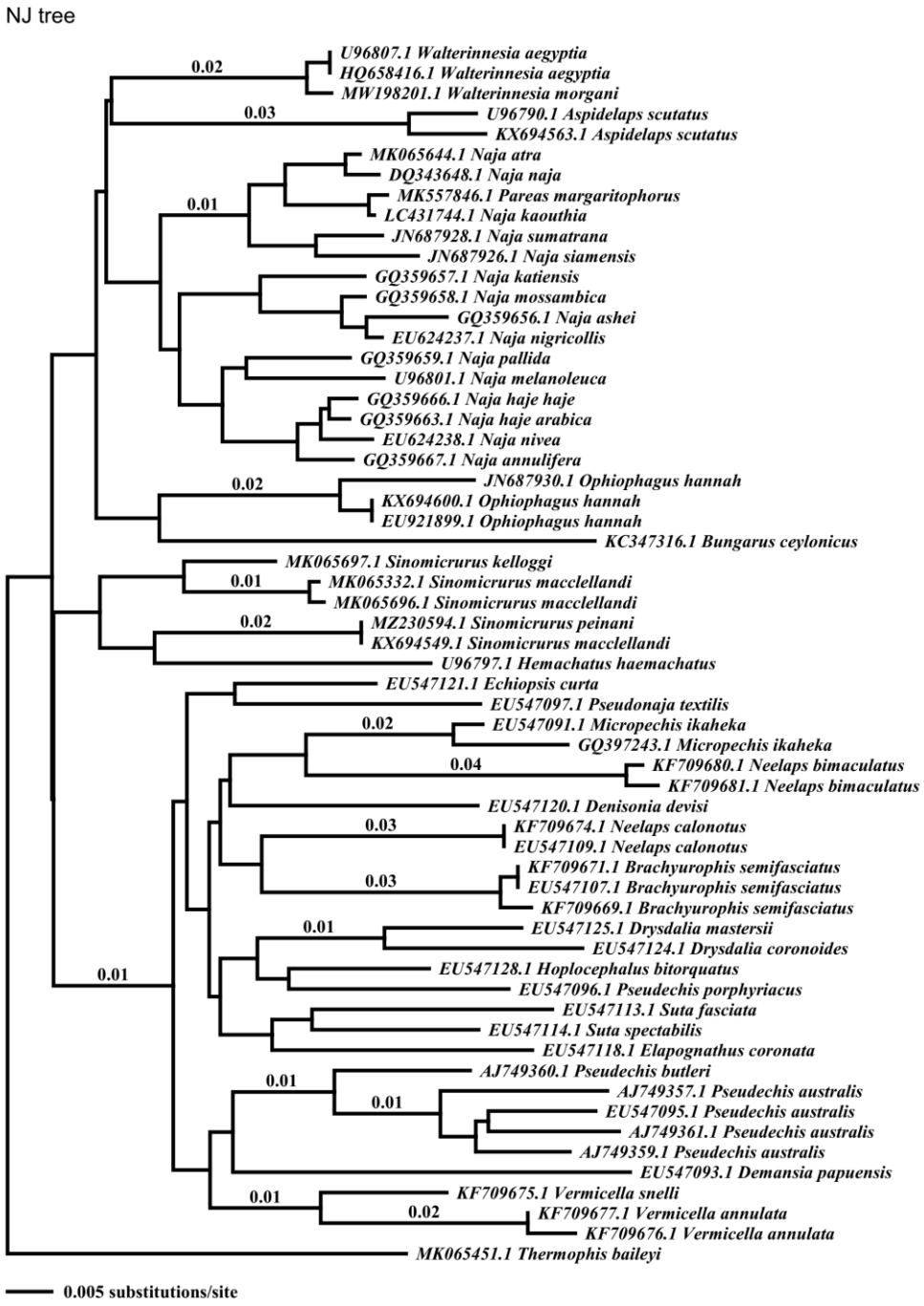

Figure 5. Neighbor-joining phylogenetic tree of genus *Walterinnesia* mtDNA sequences fragment of the 12S rRNA gene from Saudi Arabia and Egypt including Sinai, Oman, and Turkey. The number above the branches indicates distance values.