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Molecular phylogenetics of some endangered turtles reveals new close genetic relationships

Abstract. The study is devoted to detection of the phylogenetic relationships between nine specimens of turtles. Five species of them are sea turtles and included in family Chelonidae (*Caretta caretta*, *Chelonia mydas*, *Eretmochelys imbricata, Lepidochelys olivacea*) and family Dermochelyidae (*Dermochelys coriacea*). Another two species are terrestrial and placed in family Testudinidae (*Testudo kleinmanni* and *Testudo graeca*). The other turtle is fresh water turtle and grouped in family Trioychidae (*Trionyx triunguis*). The last turtle is asemiaquatic turtle and belonging to the family Emydidae (*Trachemys scripta elegans*). A total of 18 blood samples were collected from turtles in different localities of Egypt. Analyses of partial mitochondrial and nuclear sequences have revealed phylogenetic inconsistencies within family Cheloniidae and regarding the placement of *Dermochelys coriacea*. A 645 bp, 562 bp, 555 bp and 395 bp control region fragment of COI, 16S, RAG-1 and 12S was analyzed. The high percentage similarity was confirmed in GenBankfor COI, RAG-1, 16S and 12S sequences with 99%-100%. Maximum Likelihood was used to construct a phylogenetic tree. The results showed also the position of Dermochelyidae as sister to the rest of the studied marine turtles. This study succeeded in amplification by using the DNA target of the control region and it will be useful for the conservation management of marine turtles.
Key words: Cheloniidae, DNA barcoding, Nuclear gene, Phylogeny, Sequencing.

Introduction

Order Testudines are a class of reptilian that contain turtles which characterized by a special bony or cartilaginous shell developed from their ribs and acting as a shield [1]. Testudines include both live and extinct species. The earliest known species of this group date from the Middle Jurassic [2], making turtles one of the ancient reptile groups and the oldest group rather than snakes or crocodilians. Of the 356 known species alive today, some are highly endangered [3].

Dermochelyidae (Fitzinger, 1843) and Cheloniidae (Linnaeus, 1758) are two families included Seven species of marine turtles in the world. Dermochelyidae contains Leatherback *Dermochelys coriacea* (Vandelli, 1761) and Cheloniidae includes the loggerhead *Caretta caretta* (Linnaeus, 1758), the green *Chelonia mydas* (Linnaeus, 1758), the hawksbill *Eretmochelys imbricata* (Linnaeus, 1766), the Kemp's Ridley *Lepidochelys kempii* (Garman, 1880), the Olive Ridley *Lepidochelys olivacea* (Eschscholtz, 1829), Flatback *Natator depressus* (McCulloch, 1908) and Leatherback *Dermochelys coriacea* [4]. Five out of seven recognized marine turtle species are widespread in Egypt [5 – 7]. Many marine turtles are currently facing several challenges. The destruction of turtle habitat is onecauseof population decline. Additionally, humans look for eggs and turtle meat to be consumed and make souvenirs that increase the decline of turtle populations [8]. Chelonioidea contains the family Cheloniidae (5 genera, 6 species of hard-shelled sea turtles) and the monotypic leatherback marine turtle, *Dermochelys coriacea* (Dermochelyidae). Within the family Cheloniidae, hybridization was recognized, although scientific results are still scarce. Most studies focused on marine turtle hybridizationwere based solely on the description of individuals with intermediate morphological characters [9 – 12]. Recently, hybridization events have been investigated with molecular markers [13 -17]. Since the first description of a sea turtle hybrid by Garman in 1888 [14], many interspecific hybrids have been investigated using molecular markers. These studies include crossings between green turtles (*Chelonia mydas*) and hawksbills (*Eretmochelys imbricata*) [11, 14, 15], loggerheads (*Caretta caretta*) and hawksbill (*Eretmochelys imbricate*) [10, 12, 16], *Caretta caretta* and Olive Ridley sea turtle (*Lepidochelys*

olivacea) [14, 17], *Chelonia mydas* and *Caretta caretta* [14], and *Eretmochelys imbricata* and *Lepidochelys olivacea* [16]. Marine turtle hybrids are remarked between species belonging to families Carettini *(Lepidochelys olivacea*, *Caretta caretta* and *Eretmochelys imbricata*) and Chelonini (*Chelonia mydas*), whose recent phylogenetic evidence indicates a deep time divergence of about 63 million years ago.

The Loggerhead Sea Turtle (*Caretta caretta*) is the most common threatened marine turtle species in the Mediterranean Sea. Incidental fishing and increase of human activities are responsible for about 40 000 deaths per year (available from http://www.wwf.it/tartarugamarina). The International Union for Conservation of Nature listed *Caretta caretta* in the Red List of Threatened Species in spite of it is greatly studied [18, 19], little is known about the variability of some morphological characters (i.e., number of carapacial and plastron scutes) used for its recognition and if these characters are the result of a phenotypic plasticity induced by different environmental conditions.

Trionyx triunguis (Forskål, 1775) was widely distributed, relatively uniform species for decades, with no subspecies or other taxonomic units identified or proposed in recent years. Phylogeographic studies of regional variation have demonstrated different, partly conflicting results, but were based on generally limited sets of specimens and genetic markers [20-23]. However, there is still uncertainty as to whether Mediterranean and African populations may in fact represent genetically separate Management Units.

Genus *Testudo* found throughout much of the Mediterranean Sea and is represented by the smallest species of the genus, the Egyptian tortoise, *Testudo kleinmanni* (Lortet, 1883) which found in the southeastern zone. The Egyptian tortoise's range is in the northern Egypt to Cyreniaca and possibly Tripolitania in Libya [24]. Recently, Perälä [25] using solely shell morphology, argued that *Testudo kleinmanni* was in fact two species, separated by the Nile river.

Tortoises (Testudinidae: Gray, 1825) are a group of terrestrial turtles that found in Asia in the early Cenozoic (more than 60 million years ago) and then rapidly spread to Europe, Africa, and the New World [26] alongside early radiations of placental mammals [27]. Although boasting a rich fossil record [28] and diverse living members [29], the evolutionary relationships of tortoises still poorly known. Moreover, the unbeliefs about the history of tortoises are matched by doubts about their future.

Spur-thighed tortoises (*Testudo graeca* (Linnaeus, 1758)) are the most widespread species of tortoise in the Western Palaearctic. It was widely spread in three continents (Africa, Europe, Asia) and extends around 6500 km in an east-west direction from easternmost Iran to the Moroccan Atlantic coast and approximately 1600 km in a north-south direction from the Danube Delta to the Libyan Cyrenaica Peninsula. A recent report provided evidence for a deep phylogeographic structure, confirming most likely Upper Tertiary dispersal and vicariance events. Five differentiating mitochondrial lineages appear in the east of the range and at least one additional lineage in the Western Mediterranean, being sister of a Caucasian lineage [30]. Moreover, that study does not allow conclusions about phylogeographic differentiation there based on only seven samples from the Western Mediterranean. In the eastern part of the range, highly conflicting patterns were found between morphological and genetic diversity, resulting in a revised subspecies identification [30].

DNA sequence was diversity, whether evaluated directly or indirectly by protein analysis, can be used to differentiate species. More than 40 years ago, starch gel electrophoresis of proteins was first used to recognize species [31]. A single gene sequence analysis of ribosomal DNA was used to estimate evolutionary relationships at a high level from nearly 30 years ago [32], and mitochondrial DNA approaches dominated molecular systematics in the late 1970s and 1980s [33].

Turtle is considered one of the most recognizable life forms on Earth, and has more than 200 million years in the fossil record. So, key nodes in the phylogeny of turtles remain uncertain. On the other hand, Conservation attempt of marine turtles faces different challenges, as destroyed its habitat, human consumptions of meat and turtle eggs, or keep of turtle bodies as souvenirs. In their duties, conservation officers may find sea turtles in different cases (body fragments, eggs, pieces of meat, etc.), mostly it is difficult to recognize due to insufficient morphology. So, the using of DNA barcoding be an alternative for recognition at species level, contribute taxonomic and biodiversity research. This study aims to study the phylogenetic relationship of nine turtles using four different genes and provide a protocol suitable for identifying marine turtles using DNA barcoding.

Materials and methods

Collection of Samples and laboratory procedures. Eighteen specimens of turtles were collected from

different localities of Egypt. These specimens are; The loggerhead sea turtle (*Caretta caretta*) The green sea turtle (*Chelonia mydas*), The leatherback sea turtle (*Dermochelys coriacea*) The hawksbill sea turtle (*Eretmochelys imbricata*) the Olive Ridley sea turtle (*Lepidochelys olivacea*) The red-eared slider (*Trachemys scripta elegans* (Wied-Neuwied, 1839)) The African softshell turtle or Nile softshell turtle (*Trionyx triunguis*) Kleinmann's tortoise (*Testudo kleinmanni*) The Greek tortoise (*Testudo graeca*).

The first species is *Caretta caretta* is found in the Atlantic, Pacific and Indian Oceans as well as the Mediterranean Sea. It spends most of its life in habitats in salt water and estuaries [34]. The second species is *Chelonia mydas* spent most of the first five years in the Atlantic, Pacific and Indian Oceans, and young turtles are rarely seen as swimming in deep waters on the high seas as the Mediterranean Sea [30], while, the third one is *Dermochelys coriacea* is the largest of the seven species of marine turtles and turtles in general. These turtles have no real shield on their back, but their back is protected by thick leather armor and are found in all Oceans of the world and Seas [30]. The fourth species is *Eretmochelys imbricata* is an endangered sea turtle refugee living around the world and is the only species in its genus (*Eretmochelys*), followed by two different types, one is *Eretmochelys imbricata* lives in the Atlantic Ocean so, found in the Mediterranean Sea and the other is *Eretmochelys bissa* in the Pacific Ocean [35], and the fifth one is *Lepidochelys olivacea* lives in most of the world, with tropical and warm waters from the Pacific Ocean to the Indian, from the beaches of the Arabian Peninsula passing through the Red Sea, India, Japan and Micronesia to South Africa, it was also registered in various regions of the Atlantic Ocean, from the coasts of the West Africa to Brazil [30].

Trachemys scripta elegans is the sixth species which is a semi-aquatic turtle, it does not come out of water except for a specific purpose, and it is the most common species of turtle as pets throughout the whole of the world [36], moreover the seventh one is *Trionyx triunguis* which includes a large group of freshwater turtle species, native to Africa and found in the High Dam Lake in Egypt [30].

On the other hand, the eighth species is *Testudo kleinmanni* is terrestrial turtle which inhabit desert and semi-desert Egyptian environments, where they are the smallest wild turtle in the northern half of the earth, as it does not exceed the length of 10 to 15 centimeters. Egyptian turtles are exposed to great risks due to the destruction of their natural environment. These are now classified on the red list as a species that is extremely vulnerable [30]. The ninth species is *Testudo graeca*, these turtles inhabit a wide area that extends across the three continents of the ancient world, including a large part of the coasts of the Arab world on the Mediterranean and its northern regions, in addition to some neighboring countries in the Middle East, and large parts of southern and eastern European continent [30, 37, 38] (Table 1).

Table 1 – All studied species of turtles

Species	Common name	IUCN Red List status*
Caretta caretta	Loggerhead	Endangered
Chelonia mydas	Green Turtle	Endangered
Eretmochelys imbricata	Hawksbill	Critically Endangered
Lepidochelys olivacea	Pacific Ridley	Vulnerable
Dermochelys coriacea	Leatherback	Critically Endangered
Trachemys scripta elegans	Red-eared Slider Turtle	Least Concern
Trionyx triunguis	Nile Softshell Turtle	Vulnerable
Testudo graeca	Greek Tortoise	Vulnerable
Testudo kleinmanni	Egyptian Tortoise	Critically Endangered

*IUCN Red List data from http://www.iucnredlist.org/

In the current study, three samples from *Caretta caretta* were collected from the coast of Matrouh. Two specimens of *Chelonia mydas*, two specimens of *Dermochelys coriacea* and one sample of *Eretmochelys imbricata* were captured from Alexanderia coasts. Three *Lepidochelys olivacea* were collected from Safaga coast. From Giza, one samples of *Trachemys scripta elegans* was collected. One sample of *Testudo kleinmanni* captured from Elsallom and three samples of *Testudo graeca* were toke from the marker. Samples of *Trionyx triunguis* were collected from two individuals of Lake Nasser, Egypt with a permission (Fig. 1). Due to the population decline of all species, sampling of blood samples without killing animals was pursued. All locations of the study area were visited throughout four years from October 2014 to September 2018.

Figure 1 – A location map of Egypt showing the localities mentioned in the study

DNA extraction PCR amplification. DNA of 18 turtles was extracted from blood samples. These samples were collected from the studied species in an EDTA contained tube. DNA was extracted according to guidelines using the PrimePrep TM Genomic DNA Isolation Kit (GenetBio, Korea). Extracted DNA was stored at 4°C until use. PCR mixes of 15 µL included 50 ng of genomic DNA, 1U of Taq polymerase, 200 µM of dNTPs, 1X Tris-KCl buffer with 1.5 mM MgCl2, and 1 µM of each primer. The PCR enhancers and primer sequences used for each amplified locus are shown in Table 2. The amplification program consisted of3 min at 95°C, followed by 35 cycles of 30 s at 94°C, 45 s at 45–50°C, 50 s at the annealing temperature of each primer, and a final extension step of10 min at 72°C. After amplification, PCR products were checked by running in 1.5% agarose gels and stained with ethidium bromide. Successful PCR bands were cut out and purified using the QIA quick PCR purification kit from Quiagen®. The clean PCR products were sequenced using an automated sequencer following the manufacturer's protocols.

Alignment and sequence properties. All mtDNA nucleotide sequences were aligned by using Clustal W software, and identical sequences were considered the same haplotype. Using MEGA 6.0software [39], the Kimura 2-parameter distance matrix of all studied species was calculated to construct Maximum Likelihood phylogenetic tree.

Table 2 – Primer sequences used to amplify the sequences with annealing temperatures used in PCR

Primer	Sequence (5-3)	Annealing temperature (°C)	Reference
COI F	TCAACCAACCACAAAGACATTGGCAC	66	Ward et al. (2005)
COI R	TAGA CTTCTGGTGGCCAAGAATCA		Ward et al. (2005)
16S F	CCGGTCTGAACTCAGATCACG T	62.5	Palumbi et al. (1991)
16S R	CGCCTGTTTATCAAAAACAT		Palumbi et al. (1991)
12S F	AAAAAGCTTCAAACTGGGATTAGATACCCC ACT AT	68	Kocher et al. (1989)
12S R	TGACTGCAGAGGGTGACGGGCGGTGTG T		Kocher et al. (1989)
RAG-1 F	TGCACTGTGACATTGGCAA	58	Townsend et al. (2008)
RAG-1 R	GCCATTCATTTTYCGAA		Townsend et al. (2008)

Results and discussion

Eighteen COI, RAG-1, 16S and 12S sequences of all studied Testudines were compared with COI, RAG-1, 16S and 12Srecords in GenBank for *Caretta caretta*, *Chelonia mydas, Eretmochelys imbricate, Lepidochelys olivacea, Dermochelys coriacea, Trachemys scripta elegans, Trionyx triunguis, Testudo graeca and Testudo kleinmanni* GenBank provided (2, 3, 4, 11), (4, 9, 4, 12), (2, 1, 3, 13), (2, 2, 7, 3), (2, 1, 4, 3), (4, 2, 1, 2), (0, 1, 1, 1), (2, 0, 0, 0) and (2, 0, 0, 0), 16S, RAG-1 and COI sequences, respectively (Table 3).

Table 3 – List of Testudines members sequenced at mitochondrial and nuclear DNA loci (12S, 16S, RAG-1 and COI)

Species	No. sequences	Accession number	Gene
Caretta caretta	2	FJ009020, FJ009027	12S
	3	FJ009028, FJ009021. AY770545	16S
	4	JF415121, JF415120, FJ009032, FJ009025	RAG-1
	11	KU923753, KU923752, KU923751, KU923750, KU923749, KU923748, KP136711, KP136712, MN124279, GQ152888, GQ152889	COI
Chelonia mydas	4	FJ039920, FJ039927, FJ039941, FJ039948,	12S
	9	HQ377543, HQ377544, HQ377546, HQ377550, FJ039935, AY770544, FJ039921, FJ039928, FJ039942	16S
	4	JF415125, FJ039932, FJ039925, AY687907	RAG-1
	12	EU600158, EU600157, GQ152877, GQ152879, GQ152878, KF894757, KF894758, GQ152880, MN124278, GQ152882, GQ152881, KU958179	COI
Eretmochelys imbricata	2	FJ039963,FJ039970	12S
	1	FJ039964	16S
	3	JF415123, FJ039968, FJ039975	RAG-1
	13	JX571752, GQ152887, JX571754, JX571760, JX571759, JX571758, KU254594, GQ152886, GQ152885, JN991341, JN991342, JN991343 JN991344	COI

Table continuation

Species	No. sequences	Accession number	Gene
Lepidochelys olivacea	2	FJ039977, FJ039984	12S
	2	FJ039985, FJ039978	16S
	7	JF415126, JF415124, FJ039989, FJ039982, JF415128, JF415127, JF415122	RAG-1
	3	KF894766, KF894767, GQ152890	COI
Dermochelys coriacea	2	FJ039906, FJ039913	12S
	1	FJ039907	16S
	4	GU085671, FJ039918, FJ039911, AY687908	RAG-1
	3	MH273769, KU883273, GQ152876	COI
Trachemys script aelegans	4	GU477770, AB090022, FR717130, AF175340	12S
	2	HQ123497, AB090050	16S
	1	HQ442393	RAG-1
	2	MH274719, KX559044	COI
Trionyx triunguis	0	————	12S
	1	KY762050	16S
	1	Gu085681	RAG-1
	1	KP136743	COI
Testudo graeca	2	HE585813, HE585851	12S
	0	————	16S
	0	————	RAG-1
	0	————	COI
Testudo kleinmanni	2	DQ991958, AF175332	12S
	0	————	16S
	0	————	RAG-1
	0	————	COI

According to the COI, RAG-1, 16S and 12S datasets, haplotypes of the same species were always placed together in all phylogenetic reconstructions. The phylogenetic tree constructed using COI gene sequences of the studied turtle species was divided into two main clades: the first clade included the studied aquatic turtle species, while the second clade contained *Testudo kleinmanni* and *Testudo graeca* (terrestrial turtles). The first clad isgrouped into two branches; the first branch consists of *Caretta caretta*, *Lepidochelys olivacea, Chelonia mydas, Eretmochelys imbricate, Dermochelys coriacea* and *Trachemys scripta elegans*, while the second branch includes *Trionyx triunguis* (Fig. 2).

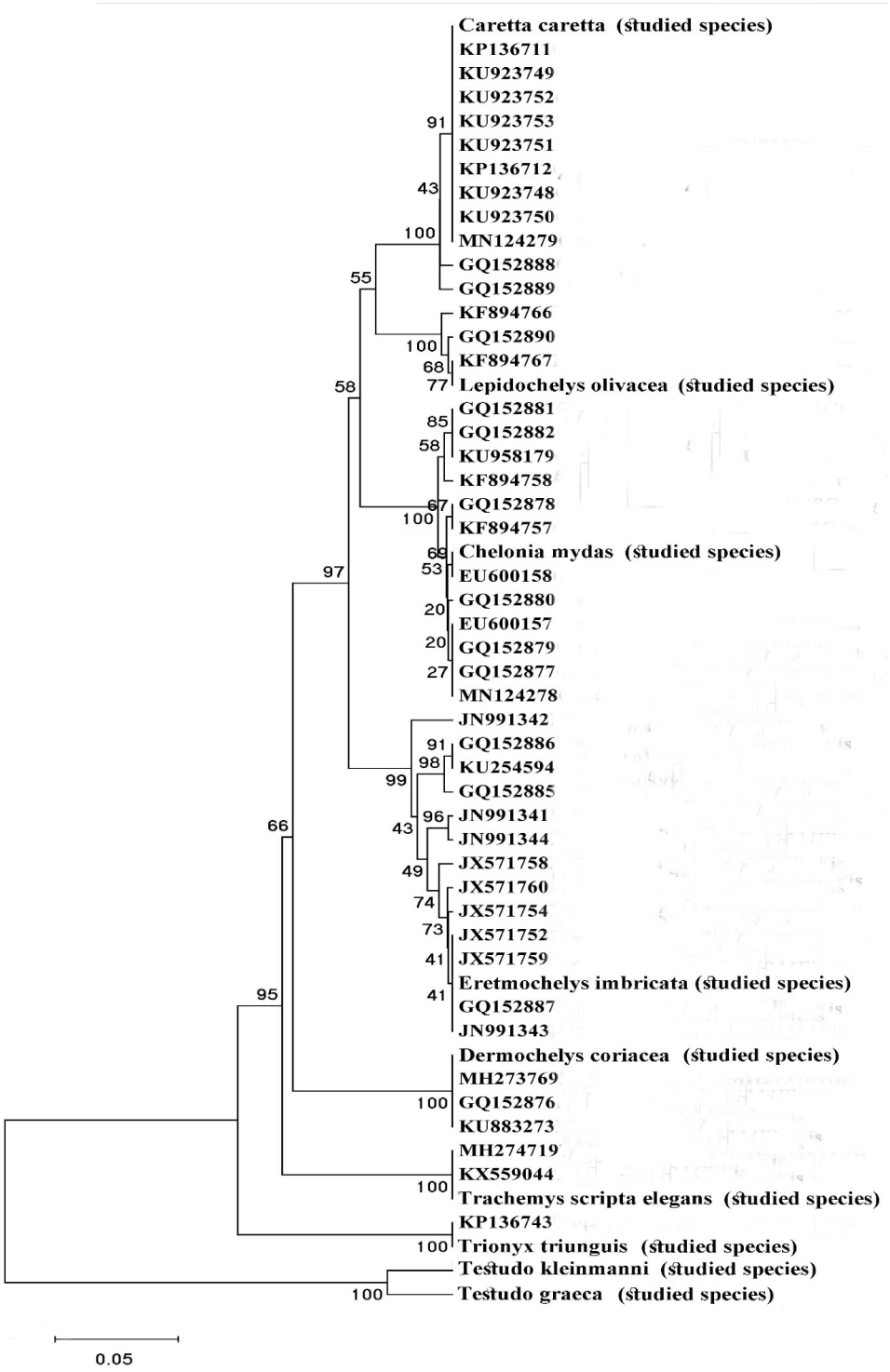

Figure 2 – Maximum Likelihood phylogenetic tree based on COI gene sequences of the studied testudines. Numbers on the branches refer to bootstrap values

Moreover, phylogenetic analysis performed in the current study using the Maximum Likelihood method revealed that *Caretta caretta* and *Lepidochelys olivacea* are closely genetically related to each other (0.072); on the other hand, *Caretta caretta* and *Testudo graeca* have the farthest genetic distance (0.614) (Table 4).

Phylogenetic tree based on RAG-1 gene sequences of the studied Testudines, was grouped into two main branches. The first branch was split into three clades. The first clade contained the members of the family Cheloniidae (*Eretmochelys imbricate*, *Lepidochelys olivacea, Chelonia mydas and Caretta caretta),* and the family Dermochelyidae (*Dermochelys coriacea*) was laid in the second clade as a sister group within Cheloniidae. The third clade included family Emydidae (*Trachemys scripta elegans*), which was grouped as monophyletic within the first and the second clades. The second branch was divided into two main clades containing the family Trionychidae (*Trionyx triunguis*), which was laid in the first main clade as a sister group within Testudinidae. The second main branch included Testudinidae (*Testudo kleinmanni and Testudo graeca*) (Fig. 3).

Table 4 – Totalgenetic distance betweenthe studied turtle species. COI distances are below diagonal; RAG-1 distances are above diagonal

	Caretta caretta	*Lepido-chelysoli-vacea*	*Eretmo-chelys im-bricata*	*Chelonia-mydas*	*Dermo-chelys co-riacea*	*Trachemys scripta el-egans*	*Trionyx triunguis*	*Testudo kleinmanni*	*Testudo graeca*
Caretta caretta		0.040	0.044	0.038	0.580	0.609	0.612	0.652	0.681
Lepidochelys olivacea	0.072		0.033	0.035	0.582	0.612	0.644	0.658	0.717
Eretmochelys imbricata	0.090	0.087		0.041	0.585	0.615	0.647	0.661	0.717
Chelonia mydas	0.084	0.077	0.069		0.582	0.603	0.638	0.655	0.684
Dermochelys coriacea	0.097	0.100	0.109	0.121		0.600	0.606	0.649	0.679
Trachemys scripta elegans	0.134	0.140	0.145	0.150	0.128		0.601	0.646	0.676
Trionyx triunguis	0.178	0.182	0.184	0.188	0.174	0.162		0.599	0.604
Testudo klein-manni	0.586	0.589	0.592	0.607	0.583	0.580	0.575		0.027
Testudo graeca	0.614	0.601	0.595	0.592	0,588	0.585	0.581	0.056	

Distances calculated between species showed that the smallest differences (0.027) existed between *Testudo kleinmanni* and *Testudo graeca*, whereas the largest was between *Testudo graeca* specimens and each of *Eretmochelys imbricate* and *Lepidochelys olivacea* (0.717, 0.717, respectively) (Table 4).

In addition, phylogenetic tree using 16S gene sequences of the studied species was constructed, which was laid into two clades. The first clade included *Caretta caretta, Lepidochelys olivacea, Eretmochelys imbricate, Chelonia mydas, Dermochelys coriacea, Trachemys scripta elegans and Trionyx triunguis* (Fig. 4), but *Testudo kleinmanni* and *Testudo graeca* were laid in the second clade, which has 0.050 as genetic distance (Table 5). Whereas the final phylogenetic tree constructed using 12S gene sequences of the studied species was split into two independent lineages, the first included all studied turtles except *Trionyx triunguis* (Figure 5).

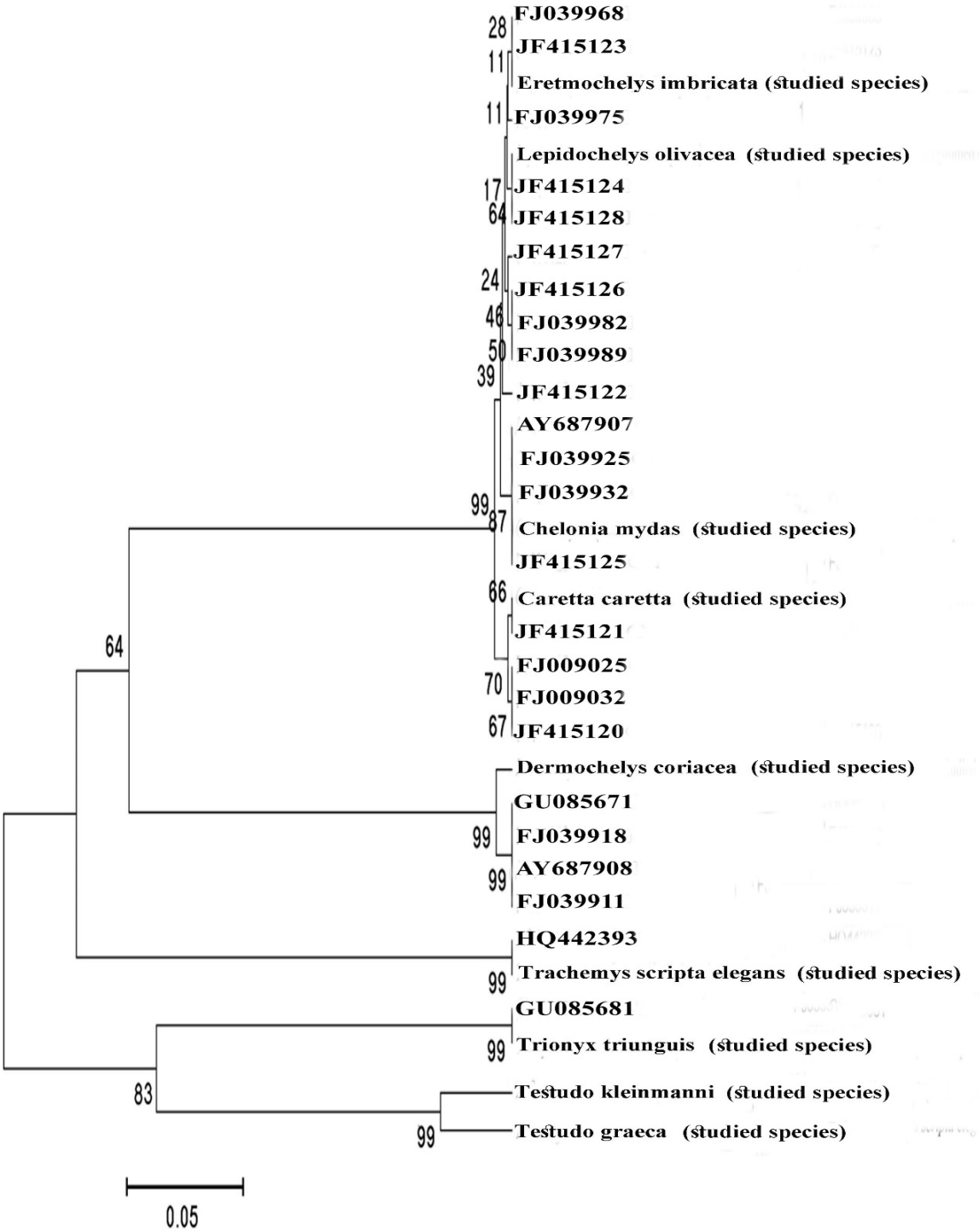

Figure 3 – Maximum Likelihood phylogenetic tree based on RAG-1 gene sequences of the studied testudines. Numbers on the branches refer to bootstrap values

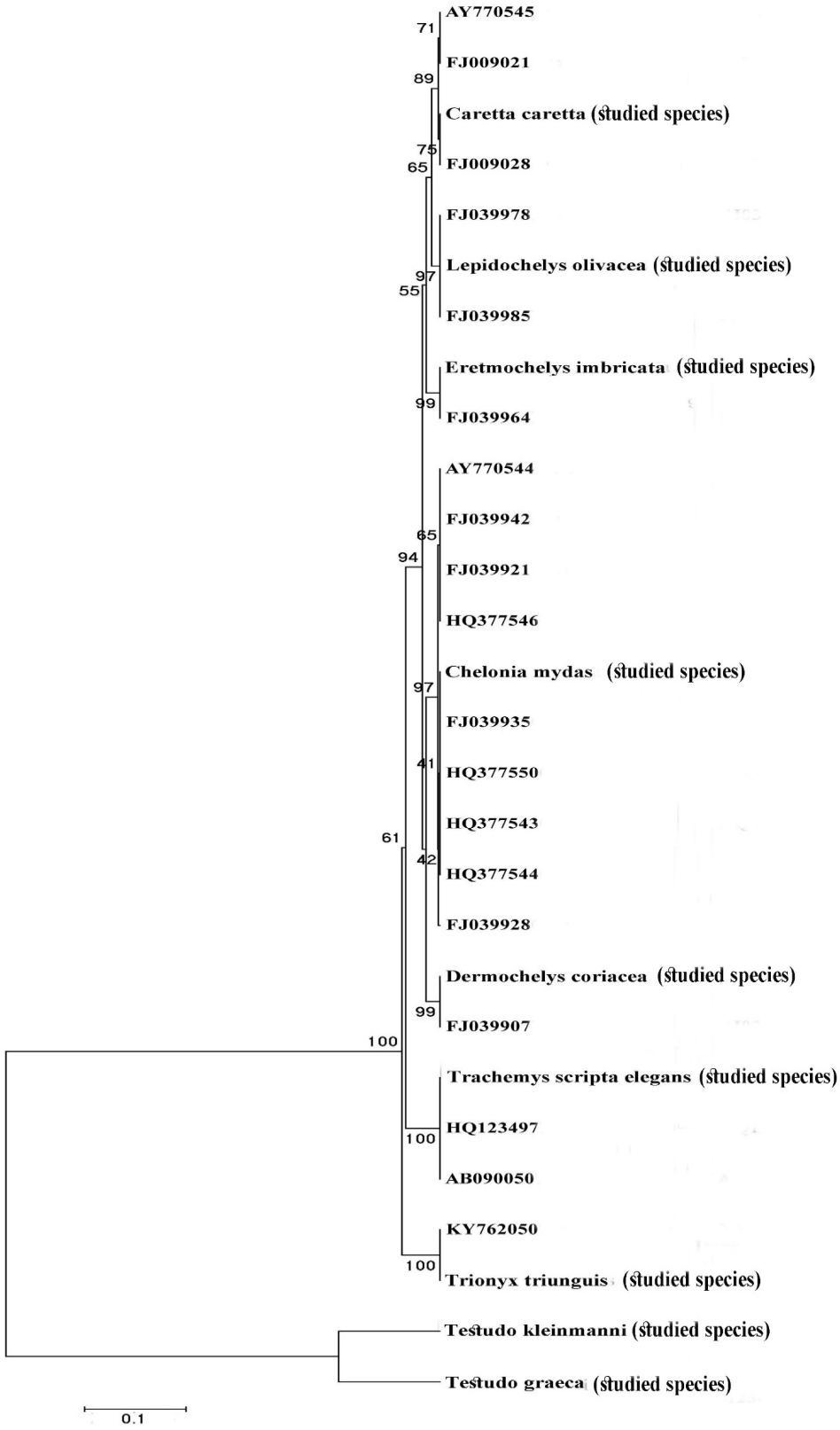

Figure 4 – Maximum Likelihood phylogenetic tree based on 16S gene sequences of the studied testudines. Numbers on the branches refer to bootstrap values

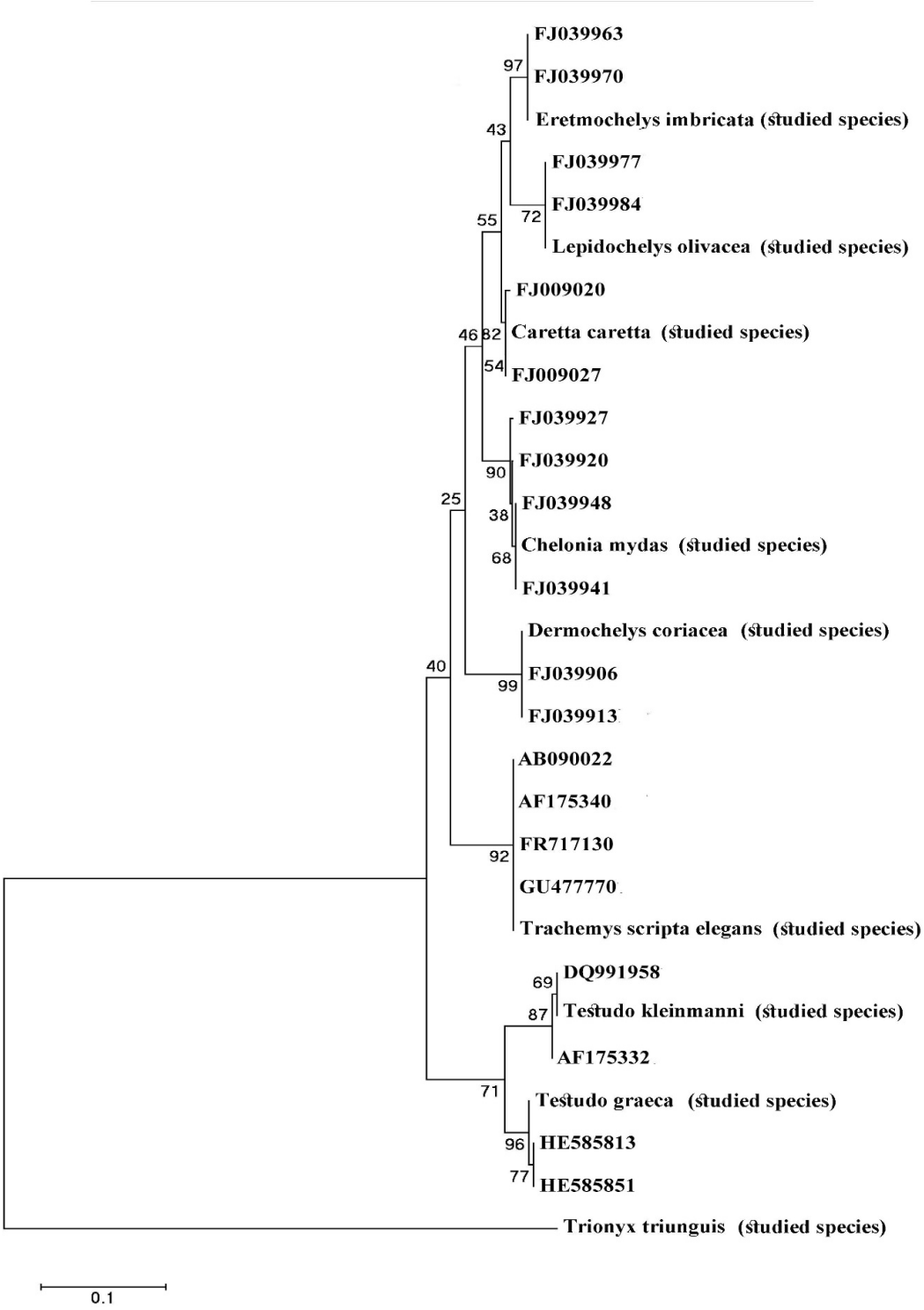

Figure 5 – Maximum Likelihood phylogenetic tree based on 12S gene sequences of the studied testudines. Numbers on the branches refer to bootstrap values

However, the smallest genetic distance between *Eretmochelys imbricate* and *Caretta caretta* was 0.030, and the largest distance between *Trionyx triunguis* and each of *Eretmochelys imbricate and Testudo graeca* was 0.615 and 0.615, respectively (Table 5).

Table 5 – Total genetic distance between the studied turtle species. 12S distances are below diagonal; 16S distances are above diagonal

	Caretta caretta	*Lepidochelys olivacea*	*Eretmochelys imbricata*	*Chelonia mydas*	*Dermochelys coriacea*	*Trachemys scripta elegans*	*Trionyx triunguis*	*Testudo kleinmanni*	*Testudo graeca*
Caretta caretta		0.022	0.037	0.040	0.049	0.274	0.395	0.615	0.628
Lepidochelys olivacea	0.044		0.028	0.040	0.049	0.274	0.395	0.612	0.628
Eretmochelys imbricata	0.030	0.043		0.037	0.046	0.271	0.380	0.609	0.625
Chelonia mydas	0.055	0.036	0.070		0.025	0.268	0.377	0.606	0.618
Dermochelys coriacea	0.079	0.089	0.092	0.072		0.265	0.362	0.606	0.618
Trachemys scripta elegans	0.109	0.114	0.119	0.095	0.082		0.358	0.603	0.615
Trionyx triunguis	0.609	0.612	0.615	0.605	0.601	0.598		0.600	0.612
Testudo kleinmanni	0.530	0.534	0.538	0.532	0.528	0.518	0.609		0.050
Testudo graeca	0.567	0.573	0.577	0.561	0.555	0.545	0.615	0.056	

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DNA barcoding is a powerful tool for species recognition and other conservation in sea turtles [40]. Generally, DNA barcoding used the COI gene. The COI target was proposed to a good candidate for barcoding animal species [41] have been successfully applied. Others researches have suggested that loci might also serve as a basic for species recognition, such as control region. Sometimes, control region sequences have been used for wildlife forensic [42]. Moreover, control region is more variable than COI to genetic population. Characteristic species control region sequences are can distinguish marine turtle species using DNA barcoding.

The recombination activase gene-1 (RAG-1) uses as a phylogenetic marker for understanding relatively deep relationships has been determined in birds [43], mammals [44] and squamates [45]. This gene is also valuable for resolving phylogenetic relationships among turtles and for resulting schisms between the models underlying the main technique used in phylogenetic analysis.

The Mitochondrially encoded 12S ribosomal RNA (mt 12S rRNA) gene was used successfully before phylogenetic questions were resolved [46 – 52]. As this gene codes for a ribosomal RNA molecule, more variation at the nucleotide level is allowed compared to a protein coding gene. The gene 16S rRNA was used for phylogeny and taxonomy; and finally, the foundation of large public-domain datasets [53, 54]. Different properties of the 16S rRNA gene make it the "Ultimate molecular chronometer", and the most common housekeeping genetic tool; and a beneficial target for clinical identification and phylogeny.

Analyzes of individual genes and the sequential data set constructed mostly well-determined, well-supported trees. The most genetic trees had similar topologies also give us with powerful trust in our resolution of many difficult deep nodes in tortoise phylogeny. Our results confirmed, with well-support, the placement of Dermochelyidae as sister to the Emydidae, a result that Bowen and Karl, [55], Dutton et al., [56] and Parham and Fastovsky, [57] recovered

from analysis of mostly complete mitochondrial genomes.

Compared to previous analyses, the results of this work differed on relationships among Cheloniidae, Dermochelyidae and Emydidae turtles. Based on both mitochondrial (mtDNA) and nuclear (nuDNA) genes, Guillon et al., [58], Iverson et al., [59] and Shaffer et al., [60] found that Cheloniidae and Dermochelyidae were sequential sister groups to Testudinidae but, Emydidae more closely related to Testudinidae. In contrast, this study reveals that Cheloniidae, Dermochelyidae and Emydidae form a monophyletic group that is sister to Testudinidae.

All tested mitochondrial and nuclear genes reveal that *Caretta caretta* is grouped within the Cheloniidae clade, as a sister taxon to the genus *Lepidochelys* of the Chelonii tribe, as expected based on literature [56, 61]. Moreover, overall tested mitochondrial and nuclear genes result that Dermochelyidae is form a monophyletic clade that is sister to the family Cheloniidae as revealed in literature [58 – 60]. The tested mitochondrial 12S and 16S genes reveal *Dermochelys coriacea* (Dermochelyidae) as a sister group with *Chelonia mydas* (Cheloniidae) as resulted in literature [59, 62, 63]. Whereas in this work, the mitochondrial COI and the nuclear RAG-1 genes result that *Dermochelys coriacea* is more closely sister taxon with *Caretta caretta*.

The family Testudinidae clade grouped as a sister taxon within the family Emydidae as reported in literature [58 – 60]. In contrast, this study results that Chelonioidea (family Chloniidae + family Dermochelyidae) clade grouped as a sister taxon within the family Emydidae. In addition, all tested genes resulted in a species tree with soft-shelled turtles, family Trionychia is form a sister clade to all other turtles.

Conclusion

The present work aims to analyze and identify turtles in Egypt using a molecular marker. In the present study, Testudines have been identified and analyzed in Egypt for the first time using DNA barcoding. These results mostly suggested that the DNA barcoding approach was used in the detection of phylogenetic relationships between the studied species. This study reveals that mitochondrial and nuclear genes were confirmed as useful and effective tools overall genes, with high power of differentiation for species identification.

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