Original

Globin-intron Sequences Imply Molecular Relationships Between the Galapagos Giant Tortoise and the Aldabra Giant Tortoise

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The author previously established a molecular tree based on the cDNA-derived primary structures of globins in two giant tortoises, *Geochelone nigra* and *G. gigantea*. The divergence time was estimated to be 15–21 million years ago. In the present study, the author reexamined the divergence time of these two species using another source of genetic information—globin-introns—including those from the Chaco tortoise (*G. chilensis*), a close relative of *G. nigra*. The previously determined divergence time was supported by the findings of this intron study. However, the inter-relationships based on the intron nucleotide sequences of the globins from the three *Geochelone* species remain controversial, because it is difficult to determine which of the three is the ancestral species. In addition, the nucleotide sequences reveal the following interesting characteristics: (1) an abnormal GC dinucleotide sequence located at the 5'-splicing site of the second intron of α^D globins instead of a consensus GT—this finding is common to all studied *Geochelone* species; (2) a repeated sequence 5'-GCCCCGCGCCCCGC-3' found only in the first intron of the *G. nigra* α^A globin gene, is a unique feature distinguishing the Galapagos giant tortoise from the other *Geochelone* tortoises that have non-repeated GCCCCGC sequences.

Key words: Aldabra giant tortoise, Chaco tortoise, Galapagos giant tortoise, globin intron, molecular relationship

アルダブラゾウガメ, チャコリクガメ, ガラパゴスゾウガメ, グロビンイントロン, 分子系統関係

(J. Nihon Univ. Med. Ass., 2013; 72 (6): 326–334)

Introduction

Only two species of giant tortoise are currently known: the Galapagos giant tortoise (*Geochelone nigra*; synonym: *Chelonoidis nigra*) and the Aldabra giant tortoise (*Geochelone gigantea*; synonym: *Dipsochelys dussumieri*), whose present habitats are restricted to oceanic islands, one being volcanic, Galapagos Archipelago^{1, 2)} and the other being an atoll, Aldabra Atoll in the Seychelles^{3, 4)}. The Galapagos Archipelago, consisting of 13 main islands, emerged about 5 to 9 million years ago (mya)²⁾. In contrast, the most recent emergence of the Aldabra Atoll occurred only 13,000 years before the present day to form a configuration of four main islands³⁾. Here, the author aims to provide scientific information on the origin and the divergence time of the two giant tortoise species, including a close relative to the Galapagos giant tortoises, the Chaco tortoise (*Geochelone chilensis*; synonym: *Chelonoidis chilensis*). To analyze intra-genus relationships, the author first constructed a molecular tree based on the primary structure of the globin subunits α^A , α^D , and β ^{5–7)}. Both giant tortoise species were shown to diverge around 15–21 mya^{6, 7)}, which represents a significant

delay following the break-up of Gondwana, which separated into Africa and South America during the Cretaceous period 65–135 mya^{8, 9)}. Hence, the author provides genetic relationships, in particular divergence times of the three *Geochelone* species, based on globin genealogy.

Globin genes consist of three exons and two introns, and exon nucleotide sequences (or primary structures) were widely used for construction of molecular relationships among living organisms^{10–13)}. On the contrary, very little is known about intron nucleotide sequences of reptilian globin⁶⁾. The author reports all intron nucleotide sequences of the globin genes, including α^A , α^D , and β in the above three species, and provides the following two molecular trees: 1) a tree based on a complete sequence of the two introns and three exons compared with a tree based on only the three exons (a protein coding sequence); 2) a tree based on only intron nucleotide sequences. The author also reveals the following two interesting structural characteristics of the introns: 1) an abnormal GC dinucleotide sequence at the 5'-splicing (donor) site of the second intron of the α^D globin instead of a consensus GT dinucleotide sequence, whose abnormal sequence was found at the 5'-splicing site in all of the

Fig. 1 Intron sequence analysis strategy

Refer to Table 1 for nucleotide sequences of the primers (F1–F9, R1–R6). Bar: 120 base pairs.

Table 1 Oligonucleotide primers used in PCR globin-intron amplification

Primer	Nucleotide sequence	Primer	Nucleotide sequence
F1	M13F-gamtatggctccgagaccctgg	R1	M13R-tggtggagggtagacgagyaac
F2	M13F-tgcgcgtggayccygtcaacttc	R2	M13R-accaccacgaagcacaggttcag
F3	M13F-gactttggggccgagggcctgg	R3	M13R-tggtggagggtagacggtgaac
F4	M13F-tgcgcgtggaccgggtcaacttc	R4	M13R-accacctggaagcwggtggacag
F5	M13F-gargtkggtggcgaagccctggc	R5	M13R-gtgggtccagggttagacgatcag
F6	M13F-tgcatgtgatccygagaacttc	R6	M13R-acgatgatgaggatrttgccag
F7	M13F-ayacatgcrggaagggaactaac	R7	M13R-ctcgtgagggttcttagcctcag
F8	M13F-ggagtggcagcaaaagcctcawg		
F9	M13F-ccaggcatgacggtgagtggtc		

M13F=GTAAAACGACGGCCAGT; M13R=CAGGAAACAGCTATGAC;

r = a or g, y = c or t, w = a or t

second introns of the α^D globins from all three *Geochelone* species; and 2) a **GCCCCGCGCCCCGC** repeat found in the first intron of the *G. nigra* α^A globin gene, which provides a practical criterion for judging the evolutionary differences between *Geochelone* species. Finally, the author provides a scenario concerning colonization of the Galapagos Archipelago and the Aldabra Atoll by the respective tortoise species.

Materials and Methods

Geochelone species

The Galapagos giant tortoise is the largest species of tortoises, whose habitat is restricted to the Galapagos Archipelago, which comprises volcanic islands about 1000 km west of Ecuador. The author used a specimen from Ueno Zoological Garden (Tokyo, Japan), which is recorded as being a donation from the late Dr. Koichi Sekiguchi (former Professor Emeritus at Tsukuba University). The Aldabra tortoise is the other extant giant tortoise, whose present habitat, the Aldabra Atoll, is located about 640 km from East Africa's coast and about 420 km from the northern tip of Madagascar. Aldabra tortoise specimens used in the present study were obtained from Osaka Municipal Tennoji Zoo (Osaka, Japan). The third, the Chaco or

Argentine tortoise, is the smallest species of land tortoises, despite been regarded as a close relative to the Galapagos giant tortoise¹⁴.

Preparation of genomic DNA

For preparation of genomic DNAs from 100% ethanol-fixed red blood cells of adult giant tortoises and the Chaco tortoises, DNA samples were prepared as described previously^{6, 7} and stored at -80°C .

Primer design and PCR conditions

Strategies for the PCR amplification of intron fragments and the design of degenerated primers are shown in Fig. 1 and Table 1, respectively. Before sequencing the PCR amplified fragments with a BigDye Terminator v. 1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), the degenerate oligonucleotide primers were tailed with the pUC/M13 forward or pUC/M13 reverse sequencing primer tail (forward 17-mer: 5'-GTAAAACGACGGCCAGT-3', Sigma-Aldrich; and reverse 17-mer: 5'-CAGGAAACAGCTATGAC-3', Promega, Tokyo, Japan). PCR amplification and the thermal profile including denaturation of the first 5 cycles were described in the previous reports^{6, 7}.

Extraction of DNA fragments, and cycle sequencing

Agarose gel (1.5%) was used to examine the purity and the size range of the PCR products amplified from the genomic DNA. The gel slices (< 500 μ g) containing the PCR products were placed into the GenElute Agarose Spin column (Sigma-Aldrich) and centrifuged for 10 min at 14,000 $\times$ g. The filtrate was concentrated using a Microcon-100 (Millipore Corp., Bedford, MA) and sequenced with an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

Using Centri-Sep spin columns (Princeton Separations P/N CS-900, Tokyo, Japan) for the removal of unincorporated dye terminations, the samples were analyzed on an ABI PRISM 310 Genetic Analyzer (Life Technologies Japan, Tokyo, Japan) as well as on an ABI 3130 Genetic Analyzer (Life Technologies).

Computer analysis

A multiple alignment program, Clustal X v. 1.81¹⁵⁾ or Clustal W v. 2.1¹⁶⁾ was used to align separately the first introns of the α^A globins and the second introns of the α^A globins. The first and second introns from the α^D globin and the β globin were similarly aligned. Phylogenetic and molecular evolutionary analyses were conducted using the Maximum Likelihood statistical method of MEGA version 5.10¹⁷⁾.

Results

Intron nucleotide sequences of α^A , α^D , and β globins

Fig. 1 shows the strategy for analyzing the intron

nucleotide sequences of globin genes from each of the three *Geochelone* species. The degenerate primers, which were deduced from exon nucleotide sequences of globin genes^{6, 7)}, are listed in Table 1. To obtain the complete nucleotide sequences of the second intron of the β globin (approximately 1600 base pairs), one or two nested PCR procedures using the nested primers listed in Table 1 were needed. The author established all nucleotide sequences of the first and second introns from each of the three *Geochelone* species. The full nucleotide sequences (Figs. S1, S2, and S3 in the Supplementary Figures), which were double-checked by forward and reverse cycle sequencing, have been deposited in gene banks under the DDBJ accession numbers listed in Table 2.

Gene tree and divergence time

Figs. 2A, 2B, and 2C, respectively show the molecular trees (gene trees) of the three *Geochelone* species revealed by the original sequence of two introns and three exons, three exons aligned as the translational unit of genes, and amino acid sequences derived from the exon nucleotide sequences. The genetic relationships of the three *Geochelone* species resulted in different branching patterns, indicating they are mutual relationships. Fig. 3 shows a genetic relationship of only introns of the three *Geochelone* species tested and constructed by the ML statistical method¹⁷⁾ whose accuracy of both the topology and branch length were tested and confirmed by several statistical methods as stored in MEGA such as Maximum-Evolutionary,

Fig. S1 Alignment of the intron structures of α^A globin. (A); 1st introns of the three species. (B); 2nd introns of the three species. Asterisks indicate invariant nucleotides. Dashed lines are gaps automatically inserted to maximize sequence alignment. Gig: *G. gigantea*, Nig: *G. nigra*, Chi: *G. chilensis*.

Fig. S2 Alignment of the intron structures of α^D globin. (A); 1st introns of the three species. (B); 2nd introns of the three species. Refer to Fig. S1 for explanations of asterisks, dashed lines, gaps, and abbreviation of species names.

Neighbor-Joining, UPGMA, and Maximum Parsimony¹⁷).

Goodman et al¹⁰) and Czelusniak et al¹¹) measured the separation time of β - α globin lineages of gnathostome and amniote (reptile-bird-mammal) ancestors to be about 300–425 mya (average time: 362.5 mya). They also estimated teleost-tetrapod to amniote α globin ancestor and tetrapod to amniote β ancestor to be 300–400 mya and 300–340 mya, respectively. Based on this assumption, the divergence times of the three *Geochelone* species were calculated to be approximately 12–20 mya (Figs. 2A and 2B, Table 3).

GC splicing donor site

Interestingly, an abnormal GC dinucleotide sequence at the 5' splicing donor site not beginning with a consensus GT sequence was detected in the second intron of α^D globin from each of the three *Geochelone* species (Fig. S2). Together with the previous finding in *G. gigantea*⁶), this finding is the second instance of abnormal splicing sites (donor/acceptor; 5'-GC/AG-3') in reptiles.

Discussion

Molecular relationships and divergence times

Based on mtDNA sequences, Caccone et al^{14, 18}) reported two divergence times for the South American tortoises and the Madagascan tortoises: one was 6–12

mya for *G. nigra* and *G. chilensis*, and the other was 14–22 mya for the four tortoises endemic to Madagascar (including two species of *Geochelone*). On the contrary, the author has established the molecular phylogeny of the three populations of *Geochelone* (one from Africa and two from South America) based on the primary structures of 53 subunits of amniote globins, including those of seven species of reptiles, three species of birds, and humans⁷). The divergence time of the Galapagos giant tortoises and the Aldabra giant tortoises was estimated to be 15–21 mya on the basis of globin genealogy^{5–7}). Even though their present habitats are separated by great distance, it was shown that both giant tortoise species diverged during the early Oligocene to the late Miocene epochs, which is supported by recent studies of the two groups^{19, 20}). To reexamine this, the author analyzed the genetic information provided by other components of the globin genes of α^A , α^D , and β , namely introns²¹). In this report, the author added the results of analysis of globin introns of a key tortoise, the Chaco tortoises.

Despite incorporating additional genetic information, the author was unable to determine which of the species is the ancestral species due to their close relationships: Analysis of the amino acid sequences (Fig. 2C) which was translated from exon nucleotide sequences resulted in good agreement with findings of a previous paper¹⁴), however, together with other rela-

tionships revealed by the complete sequence of the two introns and three exons (Fig. 2A), even only the three exons (Fig. 2B), and intron nucleotide sequences (Fig. 3) showed that they are too closely related to draw a sharp line between the three species.

Recently, the relationship between gene trees and species trees has been the focus of some attention^{22, 23}, in which a consensus is that disparities between gene trees and species trees might be related to incomplete lineage sorting²³, and gene duplication and loss²⁴. In this work, it appears that this might be playing a role. However, the author can extract useful information that the two giant tortoises including the small Chaco

tortoises separated after the breakup of Gondwana. Table 3 shows that the separation times of the three species occurred approximately 12–20 mya, which supports grossly our previous reports^{5–7}) as well as the mtDNA studies of Caccone et al^{14, 18}).

Table 2 Accession numbers of three kinds of globin genes

Species name	α^A globin	α^D globin	β globin
<i>G. gigantea</i>	AB767267	AB072353	AB767268
<i>G. chilensis</i>	AB115330	AB115331	AB115329
<i>G. nigra</i>	AB116518	AB116521	AB116524

Fig. 2 Maximum likelihood (ML) and Neighbor-joining (NJ) trees depicting genetic relationships of the three *Geochelone* species. (A) The ML tree of the original sequence of two introns and three exons, (B) The NJ tree of the exon sequences (protein coding sequences), and (C) The ML tree of the amino acid sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Evolutionary analyses were conducted the ML method based on the Tamura-Nei model³⁷) in MEGA5¹⁷). The sequences of the *Xenopus laevis* α and β globin genes were used as out-groups. Refer to Table 2 for accession numbers for globin genes of the three *Geochelone* species. Other accession numbers used in the present analysis are as follows: X14259; for α^A globin of *Xenopus laevis*, J00978; for β globin of *Xenopus laevis*, J00923; for α^A globin of *Cairina moschata*, X01831; for α^D globin of *Cairina moschata*, X15739; for β globin of *Cairina moschata*, JX911158; for α^A globin of *Oxyura ferruginea*, JX911204; for α^D globin of *Oxyura ferruginea*, JX911230; for β globin of *Oxyura ferruginea*, AB605253; for α^A globin of *Podocnemis unifilis*, AB605254; for β globin of *Podocnemis unifilis*.

Fig. 3 Maximum likelihood (ML) tree depicting genetic relationships of the three *Geochelone* species based on intron nucleotide sequences of α^A , α^D , and β globin genes. Refer to Table 2 for accession numbers for globin genes of the three *Geochelone* species.

Table 3 Divergence times of three *Geochelone* species estimated by globin genealogy

Species Name	α^A globin	
	<i>G. gigantea</i>	<i>G. nigra</i>
<i>G. gigantea</i>	mya	mya
<i>G. nigra</i>	13.1+/-4.54 (17.7)	
<i>G. chilensis</i>	13.1+/-4.54 (17.7)	9.71+/-3.92 (17.7)
Species Name	α^D globin	
	<i>G. gigantea</i>	<i>G. nigra</i>
<i>G. gigantea</i>	mya	mya
<i>G. nigra</i>	14.2+/-4.83 (15.5)	
<i>G. chilensis</i>	17.1+/-5.08 (18.8)	17.1+/-5.08 (18.8)
Species Name	β globin	
	<i>G. gigantea</i>	<i>G. nigra</i>
<i>G. gigantea</i>	mya	mya
<i>G. nigra</i>	34.2+/-10.06 (43.2)	
<i>G. chilensis</i>	34.2+/-10.06 (43.2)	11.7+/-6.13 (24.4)

Data obtained from Fig. 2A.

Data in parentheses obtained from Fig. 2B.

Migration scenario of the two giant tortoises

Initially, the giant tortoises of the Indian Ocean and those from the Galapagos were considered to be the same species (referred to as *Tortue des Indes*, *Testudo indica*, or *Testudo gigantea*). In 1834, the Galapagos giant tortoise was classified as a separate species (*Testudo nigrita*)²⁵, but it still belonged to genus *Geochelone* (FITZINGER 1835), known to contain “typical tortoises” or “terrestrial turtles”. In the 1990s, the subgenus *Chelonoidis* was proposed and South American members of *Geochelone*, including the Galapagos giant tortoises (*Geochelone nigra*) and the Chaco tortoises (*Geochelone chilensis*) were grouped

into an independent clade, *Chelonoidis*^{19,20}. On the contrary, the Aldabra giant tortoises, *Geochelone gigantea* (FITZINGER 1835), were also referred to as *Dipsochelys dussumieri* (GRAY 1831). The author, however, uses genus *Geochelone* for the three species for the decisive reasons mentioned above.

The break-up of Gondwana, resulting in the opening of the South Atlantic Ocean and the separation of land into two continents, Africa and South America, began at the end of the Jurassic period, 135 mya, and ended at the end of the Cretaceous period, 65 mya⁹). During the early Cretaceous, Madagascar separated from Africa²⁶, and the South Atlantic widened rapidly into a major ocean of approximately 1,000 km wide (about one-third of its present width), while Antarctica was still attached to South America and moved south-westward continually at a slow speed⁹). If the divergence time scaled according to hemoglobin genealogy, as reported in the literature^{10-12, 27}, is correct, then where is the origin of *G. nigra* and *G. gigantea*?

Günter¹) identified and listed the giant tortoises from the Galapagos and the Indian Ocean islands, including the Seychelles and the Mascarene (now extinct). He theorized that all the giant tortoises were descended from a single ancestral population which spread via subsided land bridges. This theory was later disproved by the understanding that the Galapagos, Seychelles, and Mascarene islands are all of recent volcanic or atoll origin and have never been linked by land bridges. However, the author supports his point of view: because there were no land bridges, the common descendants dispersed by transmarine migration²⁸). The author would like to provide the following scenario explaining the divergence time and the migration processes of the three species: After the break-up of Gondwana, the ancestral species of the two giant tortoise species and the South American land tortoise diverged around 12–20 mya (or 15–21 mya⁷) in Africa, and one ancestral population recently colonized the Galapagos Archipelago by transmarine migration through Antarctica to South America before settling in its present habitat after rafting. On the other hand, one of the other populations which evolved into the Aldabra giant tortoise remained in Africa and recently colonized the Aldabra Atoll directly, or via Madagascar. The migration process of each species suggests several settlement times after the emergence of their present islands: the Galapagos Archipelago was formed 5–9 mya²) and the Aldabra Atoll appeared approximately 13,000 years before present³). Here the author would like to provide two supporting elements. The first pertains to expansion of old world monkeys from Africa to the new world (South America and Central America) approximately 40 mya²⁹). The 53 species found in the new world (new world monkeys)

have been demonstrated to be close relatives to the old world monkeys²⁹⁾. Their routes of migration reflect the transmarine migration routes mentioned above. The second pertains to analyzing the primary structures and exon nucleotide sequences (protein coding sequences) of hemoglobin from the side-necked turtle (*Podocnemis unifilis*)³⁰⁾, in which the divergence time was tentatively measured to be 102–156 mya (according to α^A globin genealogy) and 78–123 mya (according to β globin genealogy); both point to the Cretaceous period in which Gondwana had just begun to separate. These findings might be useful for supporting not only the hemoglobin genealogy used in the present study but also the dispersion of ancestral populations of the side-necked turtles to South America via continental drift. The side-necked turtle is a freshwater turtle that exits the water only for basking or egg-laying. They do not survive well in seawater due to their skin being too weak to sustain long periods of salt exposure. The author is currently in the process of obtaining genetic information from globin genes of side-necked turtles of two Podocneminae species: one from Madagascar (*Podocnemis madagascariensis*; synonym: *Erymnochelys madagascariensis*) and the other from the Amazon River (*Podocnemis unifilis*).

Abnormal sequences at the 5' splice donor site: GC

Splicing sites of introns are highly conserved and play important roles during the processing of precursor mRNA into mature mRNA. In general, the nucleotide sequences of both intron sites, 5' GT at a splice donor site and 3' AG at a splice receptor site, are consensus sequences known as the GT/AG rule. The author first found abnormal GC dinucleotide sequences at the 5' splice site of the second intron of *G. gigantea*⁶⁾ α^D globin gene, and confirmed them without exception in the *Geochelone* species studied here. Similarly, in birds, the existence of an identical GC abnormal dinucleotide sequence at the 5' splice site has been reported in the second intron of the α^D globin from chicken³¹⁾ (*Gallus gallus*) and duck³²⁾ (*Cairina moschata*). Spingola et al³³⁾ and Wu and Krainer³⁴⁾ listed abnormal splicing sites, including GC at the 5' splice donor site in different organisms, and the author is planning to research the significance of this finding. However, these molecular findings imply that reptiles and birds have a close relationship.

A repeated sequence: 5'-GCCCCGCGCCCCGC-3'

The next interesting finding is repeated nucleotide sequences with rich C-nucleotides which are common in the intron sequences of all globin genes, in particular, a repeat 5'-GCCCCGCGCCCCGC-3' sequence can be found in the first introns of the α^A globins (Fig. S1). This is a conspicuous sequence of the *G. nigra* α^A

globin gene, which infers that *G. gigantea* and *G. chilensis* are more closely related than *G. nigra* and *G. chilensis*. This finding may also provide a key sequence for searching and evaluating the closest species to the Galapagos giant tortoises. Finally, it is worthwhile introducing a research group^{35, 36)} who has been discussing a single population from an ancestral stock that evolved into the Galapagos giant tortoise. However, this population is thought to be extinct in coastal western South America.

Conclusions

1. All introns of α^A -, α^D -, and β -globin from the three *Geochelone* species, *G. nigra*, *G. gigantea*, and *G. chilensis*, were sequenced.
2. Molecular relationships of the three *Geochelone* species were established by intron nucleotide sequences.
3. The two giant tortoises, *G. nigra* and *G. gigantea*, diversified 12–20 mya.
4. An abnormal GC at the 5' splicing site of α^D globin is reported, which supports the idea of close relationships between birds and tortoises on a molecular basis.
5. A repeated 5'-GCCCCGCGCCCCGC-3' sequence is a unique sequence for distinguishing *G. nigra* from other members of *Geochelone* species.

Acknowledgements

The author thanks the late Dr. Koichi Sekiguchi, who donated the Galapagos giant tortoise specimen to Ueno Zoological Garden, and also the Osaka Municipal Tennoji Zoo for supplying the Aldabra giant tortoise specimen.

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