**Electronic supplementary material 1**

**Tropical ancient DNA reveals relationships of the extinct Bahamian giant tortoise *Chelonoidis alburyorum***

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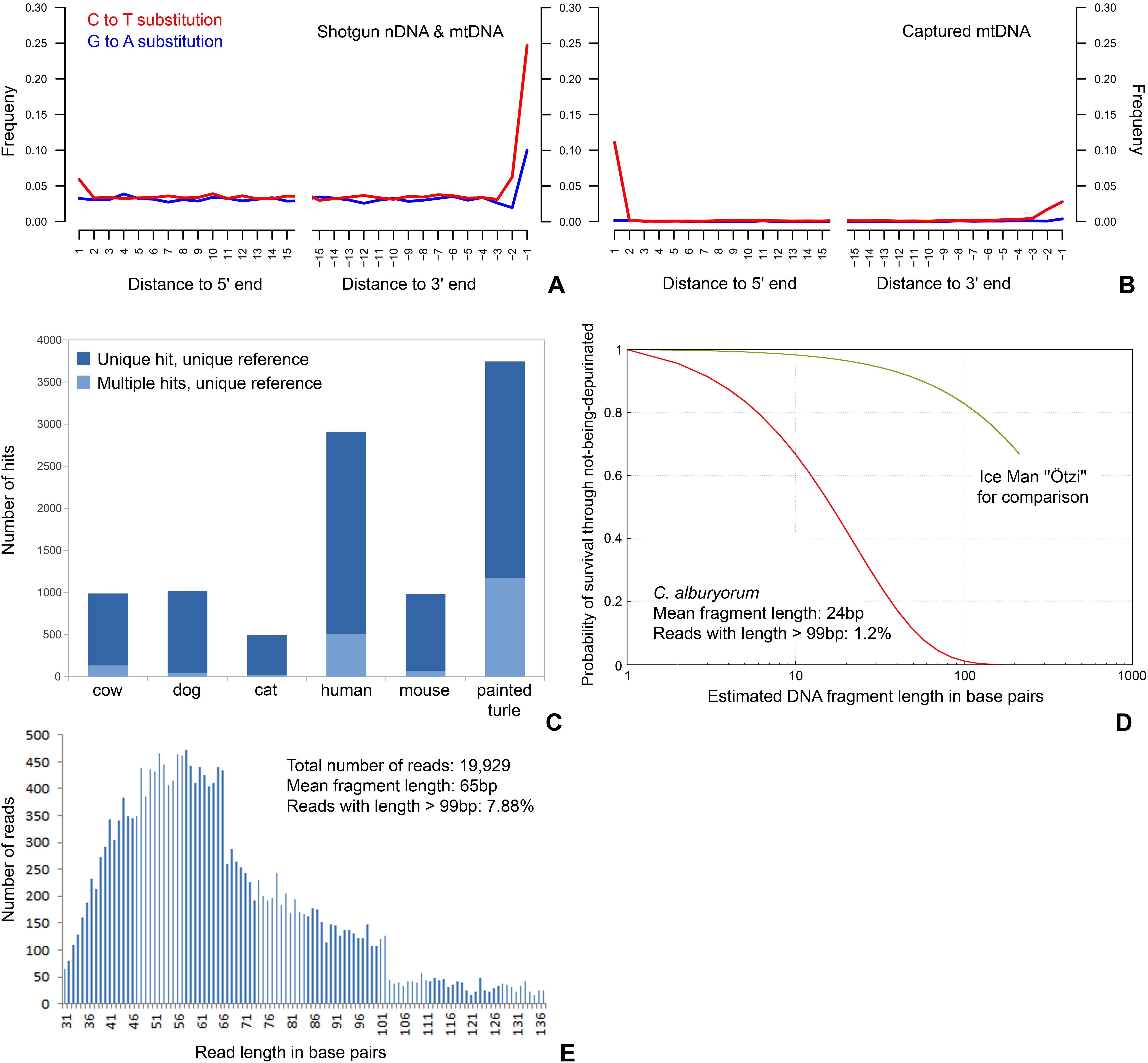
**(a) Amplicon sequencing**

Amplicon sequencing was utilised for modern samples that provided high molecular weight DNA. PCR reactions were performed in 50 μl volumes, containing 1–6 μl of DNA extract and 1 unit of *TaKaRa LA* Taq DNA Polymerase, Hot-Start Version (Clontech Laboratories Inc., Mountain View, CA, USA), following the reaction mixture recommended by the manufacturer. For primer sequences, see electronic supplementary material, table S1. PCR conditions comprised an initial denaturation at 93°C for 3 min, followed by 35 cycles of 93°C for 15 sec, 60°C for 30 sec, 68°C for 9 min, before a final elongation at 68°C for 10 min. PCR products were visualised and excised from a 2% agarose gel and purified using the peqGOLD Gel Extraction Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Only two of the three primer pairs successfully amplified the desired DNA fragments; those encompassing the control region did not amplify, probably due to the presence of large repetitive DNA blocks in the control region. Therefore, the combined long-range PCR products covered most of the mitochondrial genome from almost the beginning of 12S to almost the end of cyt *b*.

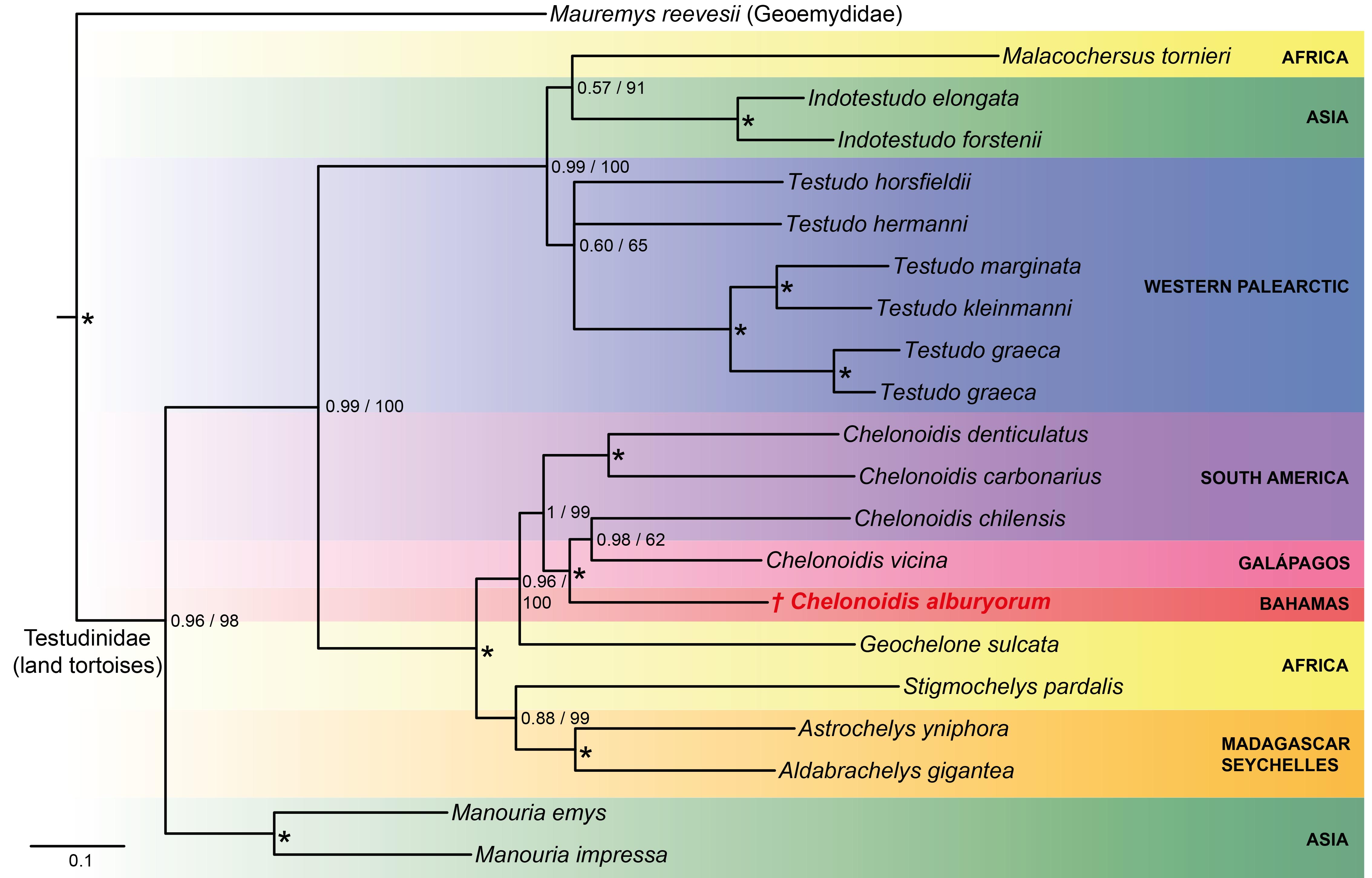
To complete the cyt *b* sequence and verify the authenticity of the long-range PCR products, this gene, in addition to 12S and ND4, was PCR-amplified and Sanger-sequenced using well-established primers (electronic supplementary material, table S1) and standard laboratory procedures (Fritz *et al.* 2014). For cycle sequencing, the total reaction volume of 10 μl contained 2 μl sequencing buffer, 1 μl premix, 0.5 μM of the respective primer, 1 μl DNA template, and ultrapure H2O. Using the ABI PRISM Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), 25 cycles were performed at 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. Reaction products were purified by gel filtration using the Performa® DTR V3 96-Well Short Plate Kit (EdgeBio, Gaithersburg, MD, USA) and 400 µl of a 5% Sephadex solution (GE Healthcare, Munich, Germany). Sequencing was performed on an ABI 3130xl Genetic Analyser (Applied Biosystems). No ambiguities were detected between the assembled complete mitogenomes and their respective Sanger-sequenced gene fragments.

**(b) Mitochondrial genomes from GenBank used for phylogenetic analyses**

All available mitochondrial genomes of tortoise species (Testudinidae) were downloaded from GenBank: *Aldabrachelys gigantea* (accession number KT613185), *Astrochelys yniphora* (JX317746), *Indotestudo elongata* (DQ080043), *I. forstenii* (DQ080044), *Malacochersus tornieri* (DQ080042), *Manouria emys* (DQ080040), *M. impressa* (EF661586), *Stigmochelys pardalis* (DQ080041), *Testudo graeca* (DQ080049 and DQ080050), *T. hermanni* (DQ080046), *T. horsfieldii* (DQ080045), *T. kleinmanni* (DQ080048), *T. marginata* (DQ080047), representing all major lineages of tortoises (Le *et al.* 2006; Fritz & Bininda-Emonds 2007). Mitochondrial genomes of the geoemydid *Mauremys reevesii* (FJ469674) and the emydid *Emys orbicularis* (JN999703) were added as outgroups. Geoemydidae and Emydidae are the successive sister groups of Testudinidae (Joyce *et al.* 2013). Since the cyt *b* gene of GenBank sequence DQ080041 has been shown to be chimerical with having a partial numt included (Fritz *et al.* 2010), the numt in DQ080041 was replaced for an authentic mitochondrial cyt *b* sequence of a *S. pardalis* (FN646157) from a collection site in close proximity (Fritz *et al.* 2010).



**Figure S1. (A)** Misincorporation plot for shotgun (pre-enriched) reads of *Chelonoidis alburyorum* mapped to the nuclear genome of the painted turtle (*Chrysemys picta bellii*). Depicted are the frequencies of substitutions in the reads relative to the reference, and their location from the 5' and the 3' read ends. **(B)** Misincorporation plot for captured reads mapped to the assembled partial mitochondrial genome of *Chelonoidis alburyorum*. (**C)** Contamination screening plot to assess endogenous DNA content in relation to potential contamination sources. Reported are number of reads that map uniquely to a given set of reference sequences. (**D)** Predicted DNA preservation for a bone sample deposited in a terrestrial environment of the Bahamas. (**E)** Fragment length distribution making up the assembled *C. alburyorum* contig.



**Figure S2.** Mitochondrial phylogeny of *Chelonoidis* species and other land tortoises inferred from Maximum Likelihood analyses using complete or nearly complete mitochondrial genomes. Outgroup (*Emys orbicularis*) removed for clarity. Numbers along nodes indicate branch support under Maximum Likelihood (1,000 bootstrap replicates) and Bayesian analyses (posterior probabilities). Asterisks indicate maximum support under both tree-building methods.

**Table S1.** PCR primer pairs and Sanger sequencing primers used in this study, including their amplified product lengths and overlap. Primers without references were designed for the present study.

|  |  |  |  |
| --- | --- | --- | --- |
| **Long-range PCR primer pairs designed for amplicon sequencing and bait-library preparation** | | **Product size** | **Overlap** |
| *Chelonoidis* spp. based on *Chelonoidis niger* (JN999704) and *Stigmochelys pardalis* (DQ080041) | |  |  |
| Chelo\_12S-ATP6\_For | 5'-GCRTACCAGTGAAAAYACCC-3' | 8,212 bp |  |
| Chelo\_12S-ATP6\_Rev | 5'-TTGGGATRAGTGGGGTTGGG-3' | 392 bp |
| Chelo\_ATP6-cytb\_For | 5'-AAGCCCACAAAYCCTWGGAA-3' | 7,141 bp |
| Chelo\_ATP6-cytb\_Rev | 5'-GGATTAGGAGRATTGTGAAGT-3' | 1,363 bp |
| Chelo\_ND6-12S\_For | 5'-AACYAACATCCCRCCCAAAT-3' | estimated 2,650 bp |
| Chelo\_ND6-12S\_Rev | 5'-TTGACCAACCCTYTTTGC-3' |  |
|  |  |  |  |
| *Geochelone sulcata* based on *G. sulcata* (KJ489404) | |  |  |
| sulcata\_12S-CO3\_For | 5'-ACAGCCCAAGACATCTAGCT-3' | 8,040 bp |  |
| sulcata\_12S-CO3\_Rev | 5'-CCTGTTAGTGGTCATGGGCT-3' | 410 bp |
| sulcata\_ATP6-cytb\_For | 5'-AGCCACAGTACTCACAGGAC-3' | 6,103 bp |
| sulcata\_ATP6-cytb\_Rev | 5'-CATCTCGGGTGATATGGGCT-3' | 92 bp |
| sulcata\_cytb-12S\_For | 5'-CCTACAAATCACCACCGGAA-3' | estimated 3,300 bp |
| sulcata\_cytb-12S\_Rev | 5'-CCTTGTTACGACTTGCCT-3' |  |
| **Internal sequencing primers for 12S and ND4** | |  | |
| 12S-L10911 | 5'-AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT-3' |  | |
| L-ND42 | 5'-GTAGAAGCCCCAATCGCAG-3' |  | |
| **Primers for amplification and Sanger sequencing of cyt *b*** | |  |  |
| CytbG3 | 5'-AACCATCGTTGTWATCAACTAC-3' | 794 bp |  |
| mt-E-Rev24 | 5'-GCRAATARRAAGTATCATTCTGG-3' | 383 bp |
| mt-c-For24 | 5'-TGAGGVCARATATCATTYTGAG-3' | 740 bp |
| mt-f-na4 | 5'-AGGGTGGAGTCTTCAGTTTTTGGTTTACAAGACCAATG-3' |  |

1 Kocher *et al.* (1989) 3 Spinks *et al.* (2004)

2 Stuart & Parham (2004) 4 Fritz *et al.* (2006)

**Table S2.** Evolutionary models according to PartitionFinder.

|  |  |  |
| --- | --- | --- |
| **Alignment position** | **Partitition** | **Model** |
| 1–69 | tRNA | JC |
| 70–2019 | non-coding | F81 |
| 2020–2090 | tRNA-Phe | K80+I |
| 2091–3087 | 12S rRNA | GTR+I+G |
| 3088–3160 | tRNA-Val | HKY+G |
| 3161–4825 | 16S rRNA | GTR+I+G |
| 4826–4907 | tRNA-Leu | K80+I+G |
| 4908–5885\3 | ND1 | HKY+I+G |
| 4909–5885\3 | ND1 | SYM+I+G |
| 4910–5885\3 | ND1 | HKY+I+G |
| 5886–6104 | tRNAs (Ile, Gln, Met) | SYM+G |
| 6105–7148\3 | ND2 | HKY+G |
| 6106–7148\3 | ND2 | HKY+G |
| 6107–7148\3 | ND2 | HKY+I+G |
| 7149–7548 | tRNA (Trp, Ala, Asn, Cyc, Tyr) | HKY+I+G |
| 7549–9142\3 | Cox1 | GTR+I+G |
| 7550–9142\3 | Cox1 | SYM+I+G |
| 7551–9142\3 | Cox1 | HKY+I |
| 9143–9282 | tRNA (Ser, Asp) | HKY+G |
| 9283–9969\3 | Cox2 | K80+I+G |
| 9284–9969\3 | Cox2 | HKY+I |
| 9285–9969\3 | Cox2 | HKY+I+G |
| 9970–10053 | tRNA-Lys | HKY+G |
| 10054–10238\3 | ATP8 | HKY+G |
| 10055–10238\3 | ATP8 | HKY+I |
| 10056–10238\3 | ATP8 | GTR+G |
| 10239–10910\3 | ATP6 | GTR+I+G |
| 10240–10910\3 | ATP6 | HKY+G |
| **Alignment position** | **Partitition** | **Model** |
| 10241–10910\3 | ATP6 | GTR+G |
| 10911–11695\3 | Cox3 | GTR+I+G |
| 10912–11695\3 | Cox3 | SYM+G |
| 10913–11695\3 | Cox3 | HKY+I |
| 11696–11783 | tRNA-Gly | HKY+G |
| 11784–12142\3 | ND3 | HKY+I+G |
| 11785–12142\3 | ND3 | HKY+G |
| 11786–12142\3 | ND3 | HKY+G |
| 12143–12216 | tRNA-Arg | HKY+G |
| 12217–12516\3 | ND4L | K80+G |
| 12218–12516\3 | ND4L | HKY+G |
| 12219–12516\3 | ND4L | GTR+I+G |
| 12517–13902\3 | ND4 | HKY+G |
| 12518–13902\3 | ND4 | HKY+I+G |
| 12519–13902\3 | ND4 | HKY+I+G |
| 13903–14138 | tRNA (His, Ser, Leu) | HKY+I+G |
| 14139–16254\3 | ND5 | HKY+I+G |
| 14140–16254\3 | ND5 | GTR+I+G |
| 14141–16254\3 | ND5 | GTR+G |
| 16255–16777\3 | ND6 | HKY+G |
| 16256–16777\3 | ND6 | HKY+G |
| 16257–16777\3 | ND6 | HKY+G |
| 16778–16847 | tRNA-Glu | HKY+G |
| 16848–17998\3 | cyt *b* | HKY+I+G |
| 16849–17998\3 | cyt *b* | HKY+I+G |
| 16850–17998\3 | cyt *b* | GTR+I+G |
| 17999–18152 | tRNA-Pro | HKY+G |
| 18153–22485 | control region | HKY+I+G |

**Table S3.** Read information of sequenced voucher specimens.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Taxon** | **Voucher** | **Laboratory method** | **# raw reads** | **# merged single reads without duplicates** | **min. read**  **length** | **ENA accession number**  **mitogenome (NGS)** | **ENA accession number**  **cyt *b* (Sanger)** |
| *Chelonoidis alburyorum* | NMB.AB50.0008 | shotgun sequencing | 4,413,742 | 1,730,143 | 20 bp | — | — |
| *Chelonoidis alburyorum* | NMB.AB50.0008 | hybridization capture | 80,053,092 | 19,907,639 | 30 bp | LT599482 | — |
| *Chelonoidis carbonarius* | MTD-T5138 | hybridization capture | 13,423,226 | 5,521,627 | 30 bp | LT599483 | LT599488 |
| *Chelonoidis chilensis* | MTD-T5754 | amplicon sequencing | 1,593,456 | 435,394 | 40 bp | LT599484 | LT599489 |
| *Chelonoidis denticulatus* | MTD-T7255 | hybridization capture | 7,667,786 | 3,099,237 | 30 bp | LT599485 | LT599490 |
| *Chelonoidis vicina* | MTD-T14174 | amplicon sequencing | 2,503,830 | 549,936 | 30 bp | LT599486 | LT599491 |
| *Geochelone sulcata* | MTD-T872 | amplicon sequencing | 2,097,698 | 454,726 | 45 bp | LT599487 | LT599492 |

**Table S4.** Observed content of endogenous tortoise DNA from a shotgun sequenced single-stranded DNA library of *Chelonoidis alburyorum*, and of the captured library and extraction blanks.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Sample** | **Laboratory method** | **Raw reads** | **Minimum read length** | **Merged reads** | **Mapped reads** | **Endogenous DNA content** | **Reference genome** |
| *C. alburyorum* | shotgun sequencing | 4,413,742 | 20 bp | 1,730,143 | 164 | 0.0095% | *Chelonoidis alburyorum*  (newly assembled) |
| *C. alburyorum* | shotgun sequencing | 4,413,742 | 20 bp | 1,730,143 | 24,362 | 1.4081% | *Chrysemys picta bellii*  (whole genome, AHGY00000000) |
| *C. alburyorum* | hybridization capture | 80,053,092 | 30 bp | 29,558,259 | 13,767 | 0.0466% | *Chelonoidis niger* (JN999704) |
| library blank 1 | hybridization capture | 3,566,976 | 30 bp | 1,124,048 | 41 | 0.0037% | *Chelonoidis niger* (JN999704) |
| library blank 2 | hybridization capture | 325,348 | 30 bp | 85,675 | 7 | 0.0082% | *Chelonoidis niger* (JN999704) |
| extraction blank 1 | hybridization capture | 1,979,628 | 30 bp | 646,034 | 10 | 0.0016% | *Chelonoidis niger* (JN999704) |
| extraction blank 2 | hybridization capture | 4,075,920 | 30 bp | 1,257,736 | 57 | 0.0045% | *Chelonoidis niger* (JN999704) |

**Table S5.** Published records of fossil *Chelonoidis* and indeterminate testudinids for the circum-Caribbean, Central America, and South America shown in Figure 2. Paleomaps in Figure 2 compiled from different sources (Iturralde-Vinent & MacPhee 1999; Scotese 2001; Iturralde-Vinent 2006; Hoorn *et al.* 2010; Roddaz *et al.* 2010; Montes *et al.* 2015), with conjectural paleorivers (Hoorn *et al.* 2010). When the *Chelonoidis* ancestor dispersed from Africa to South America, the Atlantic was roughly as wide as it is today (Scotese 2001). Equatorial paleocurrents moving then from Africa to South America (Fratantoni *et al.* 2000; Poulsen *et al.* 2001) would have created potentially favourable conditions for marine long-distance dispersal. When *C. alburyorum* diverged from *C. vicina* and *C. chilensis* in the mid-Miocene, shearing of the Caribbean plate against northern South America placed the growing Lesser Antilles island arc closer to the mainland than today (Iturralde-Vinent 2006; Xie & Mann 2014), facilitating overseas dispersal. Alternatively, the ancestor of *C. alburyorum* may have crossed into southern Central America via the proposed Panamanian connection with South America in the early middle Miocene (Montes *et al.* 2015). From there, the ancestor may have reached the Caribbean over the Yucatan Peninsula to Cuba and spread through the Greater Antillean arc.

|  |  |  |  |
| --- | --- | --- | --- |
| **Geological time** | **Taxonomic identification** | **Location** | **Sources/Remarks** |
|  |  | **Circum-Caribbean** |  |
| ? Holocene | *Chelonoidis* sp. | Eleuthera, Moore’s Island, Acklins and Mayaguana, Bahamas | Franz & Franz (2009) |
| Holocene | “*Geochelone*” sp. | Middle Caicos, Turks and Caicos Islands | Franz *et al.* (2001) |
| Holocene | “*Geochelone*” sp. | Grand Turk, Turks and Caicos Islands | Carlson (1999) |
| Late Pleistocene–Holocene | *Chelonoidis alburyorum* | Abaco, Bahamas | Steadman *et al.* (2007); Franz & Franz (2009); Steadman *et al.* (2014, 2015) |
| ? Late Pleistocene | “*Geochelone*” sp. | San Cristobal Province, Dominican Republic | Franz & Woods (1982) |
| Late Pleistocene | “*Testudo*” *monensis* | Mona Island | Williams (1952); Auffenberg (1967) |
| Late Pleistocene | “*Geochelone*” sp. | Andros and New Providence, Bahamas | Auffenberg (1967) |
| Pleistocene | “*Testudo*” *cubensis* | Cienfuegos, Sancti Spíritus and Matanzas Provinces, Cuba | Leidy (1868); Williams (1950); Auffenberg (1967) |
| Pleistocene | “*Testudo*” *sombrerensis* | Sombrero Island, Anguilla | Leidy (1868); Williams (1950); Auffenberg (1967) |
| Pleistocene | “*Geochelone*” sp. | Navassa Island | Auffenberg (1967) |
| Pleistocene | cf. “*Geochelone*” *carbonaria* | Anguilla | Cope (1883); Lazell (1993) |
| ? Pleistocene | Testudinidae | San Salvador | Olson *et al.* (1982) |
| Pleistocene | cf. “*Geochelone*” | Barbados | Ray (1964) |
| Pleistocene | “*Geochelone*” sp. | Curacao | Hooijer (1963) |
|  |  | **Central America** |  |
| Late Pleistocene | “*Geochelone*” sp. | San Miguel, El Salvador | Webb & Perrigo (1984) |
| Late Miocene | “*Geochelone*” sp. | Gracias, Honduras | Webb & Perrigo (1984) |
| Early-Middle Miocene | Testudinidae *incertae sedis* | Panama Canal Zone, Panama | Cadena *et al.* (2012) |
|  |  | **South America** |  |
| Late Pleistocene | *Chelonoidis chilensis* | Santa Fé Province, northern Argentina | de la Fuente (1997a, 1999) |
| Late Pleistocene | *Chelonoidis denticulatus* | Entre Ríos Province, northern Argentina | Manzano *et al.* (2009) |
| Late Pleistocene | “*Testudo*” *elata* | Brazil | Gervais (1877); Auffenberg (1974)  Fossils considered non-diagnostic: de Lapparent de Broin (1993); Zacarías *et al.* (2014) |
| Late Pleistocene | *Chelonoidis lutzae* | Corrientes Province, northern Argentina | Zacarías *et al.* (2013) |
| Pleistocene | “*Testudo*” *sellowi* | Paysandu, western Uruguay | Weiss (1830); Giebel (1847); Couto (1948); Auffenberg (1974) |
| Early–Middle Pliocene | *Chelonoidis australis* | Buenos Aires Province, northeastern Argentina | Moreno (1889); Rovereto (1914); Williams (1950); Auffenberg (1974); de la Fuente & Cabrera (1988); de la Fuente (1997b) |
| Early–Middle Pliocene | *Chelonoidis chilensis* | Córdoba Province, northern Argentina | de la Fuente (1988) |
| Late Miocene | *Chelonoidis gallardoi* | Catamarca Province, northern Argentina | Rovereto (1914); Auffenberg (1974); de la Fuente (1988, 1997a) |
| Middle Miocene | *Chelonoidis* sp. | Huila, central Colombia | Wood (1997) |
| Middle Miocene | *Chelonoidis hesternus* | Huila, central Colombia | Auffenberg (1971); de la Fuente (2014) |
| Middle Miocene | cf. *Chelonoidis* | Quebrada Honda, southern Bolivia | Cadena *et al.* (2015) |
| Early–Middle Miocene | *Chelonoidis* sp. | Alta Guajira, northern Colombia | Cadena & Jaramillo (2015) |
| Early Miocene | *Chelonoidis gringorum* | Chubut Province, central Argentina | Simpson (1942); Loveridge & Williams (1957); Auffenberg (1971, 1974); de la Fuente (1988, 1994) |
| Late Oligocene | ? *Chelonoidis* sp. | Quebrada Saguayo, Central Bolivia | de Broin (1991) |

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