

PALEONTOLOGY

Ancient DNA elucidates the lost world of western Indian Ocean giant tortoises and reveals a new extinct species from Madagascar

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Before humans arrived, giant tortoises occurred on many western Indian Ocean islands. We combined ancient DNA, phylogenetic, ancestral range, and molecular clock analyses with radiocarbon and paleogeographic evidence to decipher their diversity and biogeography. Using a mitogenomic time tree, we propose that the ancestor of the extinct Mascarene tortoises spread from Africa in the Eocene to now-sunken islands northeast of Madagascar. From these islands, the Mascarenes were repeatedly colonized. Another out-of-Africa dispersal (latest Eocene/Oligocene) produced on Madagascar giant, large, and small tortoise species. Two giant and one large species disappeared c. 1000 to 600 years ago, the latter described here as new to science using nuclear and mitochondrial DNA. From Madagascar, the Granitic Seychelles were colonized (Early Pliocene) and from there, repeatedly Aldabra (Late Pleistocene). The Granitic Seychelles populations were eradicated and later reintroduced from Aldabra. Our results underline that integrating ancient DNA data into a multi-evidence framework substantially enhances the knowledge of the past diversity of island faunas.

INTRODUCTION

Before human arrival, the ecosystems of the oceanic islands in the western Indian Ocean were dominated by giant tortoises as equivalents of continental megafauna (1), both in biomass and numbers (2, 3). On Madagascar, they shared that role with medium- to large-sized mammals and birds, while giant tortoises were the only large herbivores on Aldabra, the eastern Granitic Seychelles, and the Mascarene islands (3, 4). Aldabra, a coralline atoll in the extreme western Seychelles, is the only island with a surviving native giant tortoise population. Notably, the biomass of the local species (*Alidabrachelys gigantea*) reaches up to 58 tons/km², exceeding the combined biomass per square kilometer of large mammal herbivores in Africa (2, 5). Tortoises on Aldabra have been estimated to consume, on average, almost 12 million kg of plant matter per year (6), underlining the importance of giant tortoises in island ecosystems and suggesting a major disturbance of ecosystem functions after their demise elsewhere.

Not least due to their unusual appearance and sheer size of up to 138 cm straight carapace length (SCL) and body masses of 200 kg (7, 8), western Indian Ocean giant tortoises have also captured the attention of naturalists since the 17th century, when the Mascarene archipelago, the Granitic Seychelles, and Aldabra still hosted large populations (3). By then, though, Madagascar's giant tortoises had long since vanished. The two currently recognized Malagasy species became extinct after the colonization of the island by Indo-Malay people, possibly correlated with the spread of pastoralism (9). The youngest specimens of the Malagasy *Al. grandidieri* were radiocarbon-dated to 1282 to 1066 calibrated years before the present (cal yr B.P.) and those of *Al. abrupta* to 720 to 635 cal yr B.P. (4, 9, 10). However, the presumed species-specific differences between *Al. abrupta* and *Al. grandidieri* mainly relate to carapace shape (11, 12), which is known to be highly variable in chelonians (13), and there is no genetic evidence for their distinctness.

The giant tortoises endemic to the Mascarene archipelago, approximately 700 km east of Madagascar, were only extirpated between 1735 and 1840 following the arrival of Europeans (3, 8). The giant tortoise populations on the Granitic Seychelles experienced a similar fate and presumably went extinct in the early 19th century (3). However, on the basis of the morphological similarity of captive tortoises and museum specimens from the 19th century thought to originate from the Granitic Seychelles, the survival of a few individuals from these islands has been suggested (8, 14, 15). Only Aldabra, the westernmost island in the Seychelles and 450 km from northern Madagascar, still harbors a dense population of giant tortoises. The atoll is now a strictly protected UNESCO World Heritage Site with approximately 100,000 tortoises (16).

The advent of ancient DNA (aDNA) approaches led to first attempts to elucidate the diversity and evolutionary history of the extinct giant tortoises from the western Indian Ocean. Using Sanger-sequenced short fragments of mitochondrial DNA

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(mtDNA), the distinctiveness of five Mascarene giant tortoise species of the genus *Cylindraspis* was confirmed (*C. indica* from Réunion, *C. inepta* and *C. triserrata* from Mauritius, and *C. peltastes* and *C. vosmaeri* from Rodrigues) (17). However, their phylogenetic and biogeographic relationships could not be established. In addition, a single short mtDNA fragment of the extinct *Al. grandidieri* from Madagascar was found to be different from the extant *Al. gigantea* from Aldabra. Sequencing *Al. abrupta*, the second extinct Malagasy species, failed (18), leaving unresolved the old speculation of whether *Al. abrupta* could be conspecific with the extant species from Aldabra (11).

On the basis of morphological variation of historical museum specimens and extant (mostly captive) tortoises, three distinct *Aldabrachelys* species were proposed for the Granitic Seychelles (*Al. arnoldi*, *Al. daudinii*, and *Al. hololissa*) (15). However, using 428–base pair (bp)–long mtDNA sequences from subfossil material, historical museum specimens, and some living tortoises from Aldabra and possibly the Granitic Seychelles, only minimal genetic variation was revealed (18), a finding supported by genome-wide population structure analyses of extant Aldabra tortoises (19). The lack of divergence for extant tortoises from these islands was supported in another study based on eight microsatellite loci and mitochondrial control region sequences (20), suggesting that only a single *Aldabrachelys* species survived. Consequently, the giant tortoises from the Granitic Seychelles have since been considered conspecific with those from Aldabra but assigned to subspecies that are thought to be either extinct (*Al. gigantea daudinii*) or to survive in low numbers in captivity and in introduced feral populations (*Al. g. arnoldi* and *Al. g. hololissa*) (8).

For the five extinct Mascarene *Cylindraspis* species, a recent investigation revealed a complex biogeography (21). Using near-complete mitochondrial genomes (mitogenomes) and paleogeographic reconstructions, it was shown that these animals represented a deeply divergent clade of Eocene origin that was distantly related to all other tortoise species, including the other giants from the western Indian Ocean. The diversification of *Cylindraspis* had already commenced in the Oligocene, long before the Mascarenes surfaced, most likely on a series of now-submerged volcanic islands of the Réunion hot spot north of the Mascarenes. Most likely, *Cylindraspis* repeatedly dispersed from there to the Mascarenes, circumventing Madagascar and the Seychelles Plateau. Several subsequent colonization pulses from the north and within the Mascarenes led to the occurrence of two distinct *Cylindraspis* species both on Mauritius and Rodrigues, despite the very small size of these islands (1845 and 104 km²) that make in situ divergence unlikely (21).

The present study aims at clarifying the diversity and biogeography of giant tortoises based on remains from western Indian Ocean islands using contemporary aDNA approaches. We generated near-complete mitogenomes for subfossil tortoise material from Madagascar and the Granitic Seychelles and historical museum specimens collected 300 to 200 years ago from the Mascarenes and presumably the Granitic Seychelles. We combine the sequences with data for most of the extant tortoise lineages plus some extinct taxa and with evidence from paleogeography, radiocarbon-dated subfossil material, and nuclear aDNA to reconstruct the biogeography and original diversity of the western Indian Ocean tortoise fauna. In doing so, we discover and describe a previously unknown extinct large-sized tortoise species from

Madagascar as new to science and propose a comprehensive dispersal scenario for all western Indian Ocean tortoise species. For inferring hypothetical dispersal routes of maternal tortoise lineages, we use the phylogeny of mitogenomes. We are well aware that the true species tree of testudinids may differ from their maternal mitochondrial gene tree because of nuclear genomic differences caused by male-mediated gene flow, recombination, and introgressive hybridization. However, we expect these factors to play only a minor role for the geographically isolated island taxa, which are in the focus of our biogeographic reconstructions.

RESULTS

We sequenced near-complete mitogenomes from three subfossil tortoises morphologically identified as *Al. abrupta* (Madagascar), from five subfossil specimens identified as *Al. grandidieri* (Madagascar), and from two subfossil *Aldabrachelys* evidently or presumably from the Granitic Seychelles (average coverage ranging from 17- to 523-fold; table S1). Among these specimens were the name-bearing lectotype of *Al. abrupta* and the paralectotype of *Al. grandidieri*. Sequencing 19 additional subfossil specimens failed (table S2), including the lectotype of *Al. grandidieri*, which was excavated from the same site as the paralectotype (12).

Furthermore, 12 historical museum specimens of *Aldabrachelys* and 2 *Cylindraspis* specimens were studied, including many types. The museum specimens of *Aldabrachelys* included all specimens from the natural history museums in London [Natural History Museum United Kingdom (NHMUK)] and Paris [Muséum national d'Histoire naturelle (MNHN)] that are thought to represent native tortoises from the Granitic Seychelles before their extinction or near extinction. Among them was type material of the three currently recognized subspecies of *Al. gigantea* from the Granitic Seychelles (*Al. g. arnoldi*, *Al. g. daudinii*, and *Al. g. hololissa*). Near-complete mitogenomes could be generated for all historical specimens (average coverage 255- to 4789-fold). Both *Cylindraspis* specimens yielded near-complete mitogenomes, with a 606- and 2686-fold average coverage, respectively. One of these specimens is the holotype of *Testudo schweigeri*, and the other is one of the two only *Cylindraspis* hatchlings known to exist [table S1; see there also for European Nucleotide Archive (ENA) accession numbers of all newly generated mitogenomes]. In addition, sequencing seven variable nuclear loci (22) was tried for the same subfossil material from Madagascar, which yielded mtDNA genomes, and for the two *Cylindraspis* samples. However, nuclear sequences could be obtained only for four loci of one specimen morphologically determined as *Al. abrupta* (MNHN.F.MAD480; 3047 bp; average coverage 18- to 44-fold), underlining the challenges of sequencing nuclear DNA from ancient material from tropical environments.

Our phylogenetic, biogeographic, and molecular clock analyses for mitogenomes also included previously published data for representatives of all genera and species groups of extant tortoises and of the extinct giant tortoises from the Mascarenes (*Cylindraspis*) and Bahamas (*Chelonoidis alburyorum*) (21, 23, 24). Additional phylogenetic analyses were run for a similar nuclear dataset lacking the extinct *Cylindraspis* and *Chelonoidis* species and, except for one sample, the extinct Malagasy tortoises (table S3). The general results for the mitochondrial and nuclear datasets (Figs. 1 to 3 and fig. S1) were similar and conform to previous studies (21–25).

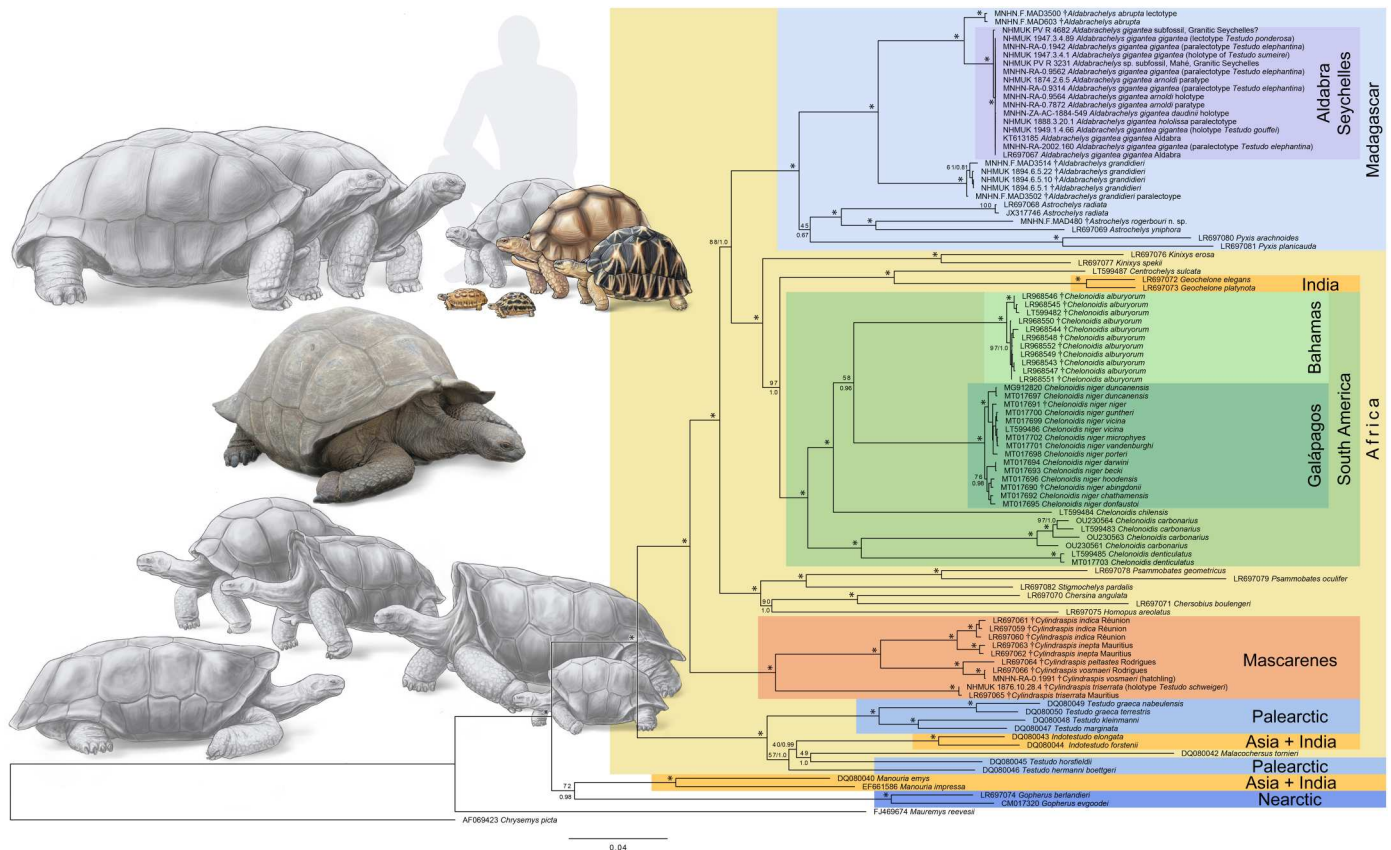


Fig. 1. Placement of extinct Malagasy and Mascarene giant tortoises in the tortoise phylogeny. The shown maximum likelihood (ML) topology is based on near-complete mitochondrial genomes (15,537 bp) of all genera and species groups of tortoises (Testudinidae). Codes preceding scientific names are DNA Data Bank of Japan (DDJB)/European Nucleotide Archive (ENA)/GenBank accession numbers or museum numbers. Genus names of extinct taxa bear dagger symbols. Numbers at nodes are thorough bootstrap values and posterior probabilities from a Bayesian analysis yielding the same topology. Asterisks indicate maximum support under both approaches. Colored boxes represent different geographic regions. Inset pictures show the past and present diversity of native tortoise species of the western Indian Ocean (extinct species are in gray). Top: Madagascar, from left to right, †*Aldabrachelys abrupta*, †*Al. grandidieri*, *Pyxis planicauda*, *P. arachnoides*, †*Astrochelys rogerbouri* n. sp., *As. yniphora*, and *As. radiata*. Center: Granitic Seychelles (extinct), Aldabra, *Al. gigantea*. Bottom: Mascarenes, from left to right, †*Cylindraspis indica* (Réunion), †*C. inepta*, †*C. triseriata* (both Mauritius), †*C. vosmaeri*, and †*C. peltastes* (both Rodrigues). Sizes to scale, corresponding to reported maximum SCLs (8). Artwork: Michal Rössler; photo of *Al. gigantea*: Massimo Delfino.

According to the mitochondrial analyses, the extinct *Cylindraspis* species represented a deeply divergent clade of Eocene age [39.1 million years (Ma) ago; Fig. 2] that was sister to a major clade comprising a diverse assemblage of tortoise genera from Africa, Madagascar, the Granitic Seychelles and Aldabra, India, South America, Galápagos, and the Bahamas (Fig. 1), mirroring the out-of-Africa dispersal of that clade (21).

All studied *Aldabrachelys* samples, except for one identified as *Al. abrupta* (MNHN.F.MAD480), clustered in a maximally supported clade that also contained extant *Al. gigantea* from Aldabra. This *Aldabrachelys* clade was nested within a more inclusive clade in which all extant native tortoises from Madagascar (*Astrochelys radiata*, *As. yniphora*, *Pyxis arachnoides*, and *P. planicauda*) and the sequence from MNHN.F.MAD480 were placed (see below). This Madagascar-Aldabra/Seychelles (MAS) clade diverged from its sister group containing tortoise genera from Africa, India, South America, Galápagos, and the Bahamas 34.3 Ma ago (latest Eocene), with a 95% highest posterior density interval reaching well into the Middle Oligocene (Fig. 2).

Within the MAS clade, three maximally supported subclades of *Aldabrachelys* were recovered (Fig. 1). One corresponded to the mitogenomes of the five samples of *Al. grandidieri*, including the paralectotype of this species (MNHN.F.MAD3502). According to our molecular clock (Fig. 2), *Al. grandidieri* branched off from its sister group in the Middle Miocene (15.7 Ma ago). Its sister group included two clades, which diverged 4.5 Ma ago. One of these two clades contained the mitogenomes of the lectotype of *Al. abrupta* (MNHN.F.MAD3500) and another subfossil specimen identified as *Al. abrupta* from the same excavation site. This *Al. abrupta* clade was sister to a shallow clade containing the sequences of all historical museum specimens of *Aldabrachelys* and two subfossil samples that presumably (NHMUK PV R 4682) (11) or evidently (NHMUK PV R 3231, Mahé, Anse Royale) (18) originated from the Granitic Seychelles plus two mitogenomes of extant *Al. gigantea* from Aldabra. Variation occurred both among the mitogenomes of *Al. abrupta* and *Al. grandidieri*, with our molecular clock estimating that the divergence of matrilineages in *Al. grandidieri* commenced 1.1 Ma ago. Although only two mitogenomes of *Al. abrupta* were

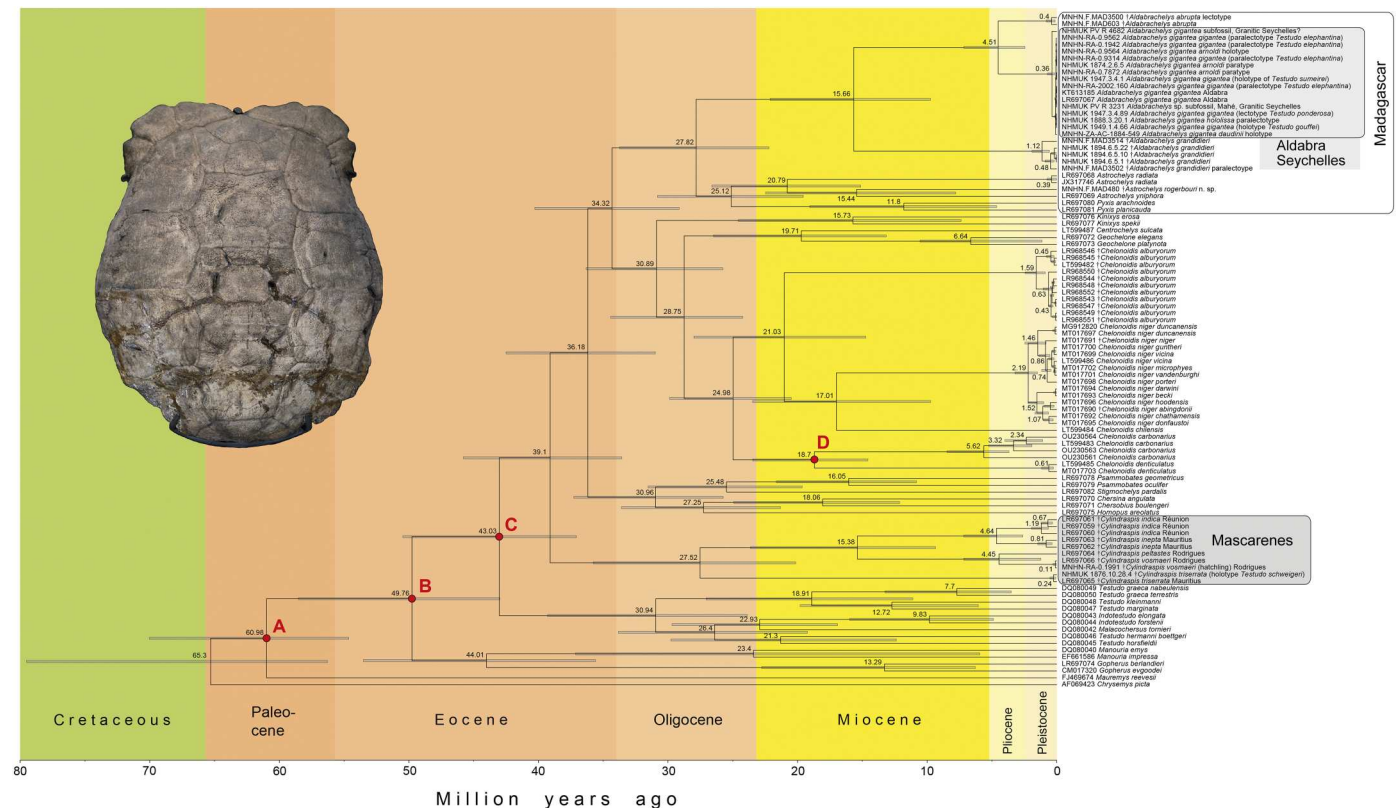


Fig. 2. Fossil-calibrated time tree for tortoises (Testudinidae). This analysis is based on the same dataset as that presented in Fig. 1. For each node, estimated mean ages and 95% highest posterior density intervals are shown. Codes preceding scientific names are DDJB/ENA/GenBank accession numbers or museum numbers. Genus names of extinct taxa bear dagger symbols. The red circles indicate fossil calibration points (see detailed explanation in the Supplementary Materials): (A) *Hadrianus majusculus*, 100.5 to 50.3 Ma ago; (B) *Cheirogaster maurini* and *Gigantochersina ammon*, 66.0 to 33.9 Ma ago; (C) *Cheirogaster maurini*, 47.8 to 33.9 Ma ago; and (D) *Chelonoidis hesternus*, 33.9 to 11.8 Ma ago. Inset: *Aldabrachelys grandidieri*, paralectotype (MNHN.F.MAD3502; photo: Nour-Eddine Jalil).

available, their estimated divergence age of 400,000 years ago still exceeded that of the third clade containing the mitogenomes of *Aldabrachelys* from Aldabra and from historical and subfossil material from the Granitic Seychelles. In 15 of the 16 mitogenomes in this clade, virtually no sequence variation was detected (Fig. 1), and only the mitogenome of one subfossil specimen (NHMUK PV R 4682) of possible Granitic Seychelles origin (11) was slightly distinct, with an estimated divergence time of 360,000 years ago.

Unexpectedly, the mitogenome sequenced from a tibia (MNHN.F.MAD480) excavated at the type locality of *Al. abrupta* (Ambolisatra in extreme southwestern Madagascar) and morphologically identified as a juvenile of this species (12) was positioned outside of *Aldabrachelys*. It was placed as the deeply divergent sister lineage of *As. yniphora*, a critically endangered extant species from northwestern Madagascar (8). Our molecular clock analysis inferred that the mitogenomes of MNHN.F.MAD480 and *As. yniphora* diverged 15.4 Ma ago, i.e., approximately at the same time when the mitochondrial lineage of *Al. grandidieri* branched off from its sister clade (*Al. abrupta* + *Al. gigantea*) and substantially before the matrilineages of the extant Malagasy tortoise species *P. arachnoides* and *P. planicauda* separated (11.8 Ma ago).

The two new *Cylindraspis* mitogenomes closely agree with our previously published sequences for this genus (21). The mitogenome of a species-undetermined hatchling from Rodrigues

(MNHN-RA-0.1991) clustered with *C. vosmaeri*, providing evidence that this specimen, one of the only two complete alcohol-preserved *Cylindraspis* hatchlings (26), represents this species and not the second *Cylindraspis* species from Rodrigues, *C. peltastes*. Our sequence from the type specimen of *T. schweigeri* (NHMUK 1876.10.28.4, juvenile of unknown provenance) clustered with a mitogenome from a *C. triserrata* from Mauritius, confirming earlier conclusions about the identity of *T. schweigeri* based on a short mtDNA fragment (17, 26). NHMUK 1876.10.28.4 is the only known complete *C. triserrata* shell with epidermal scutes; all other specimens only consist of bones or bony shell fragments (26). *Cylindraspis triserrata* became extinct c. 1735 (8, 10).

The phylogenetic trees using our nuclear dataset (Fig. 3) were largely concordant with the mitochondrial results (Fig. 1), but many deeper branching patterns were less resolved. Other differences referred mainly to the placement of the African genus *Kinixys* and the relationships within the *Testudo-Indotestudo-Malacochersus* clade. Notably, the terminal branching patterns of our nuclear tree matched well a recently published analysis based on 15 nuclear loci for the extant species (22), supporting that the most variable loci chosen by us from this dataset reliably resolve species divergences, despite representing only a small fraction of the nuclear genome. As in our analyses of mitogenomes, sample MNHN.F.MAD480 was again with high support the sister lineage

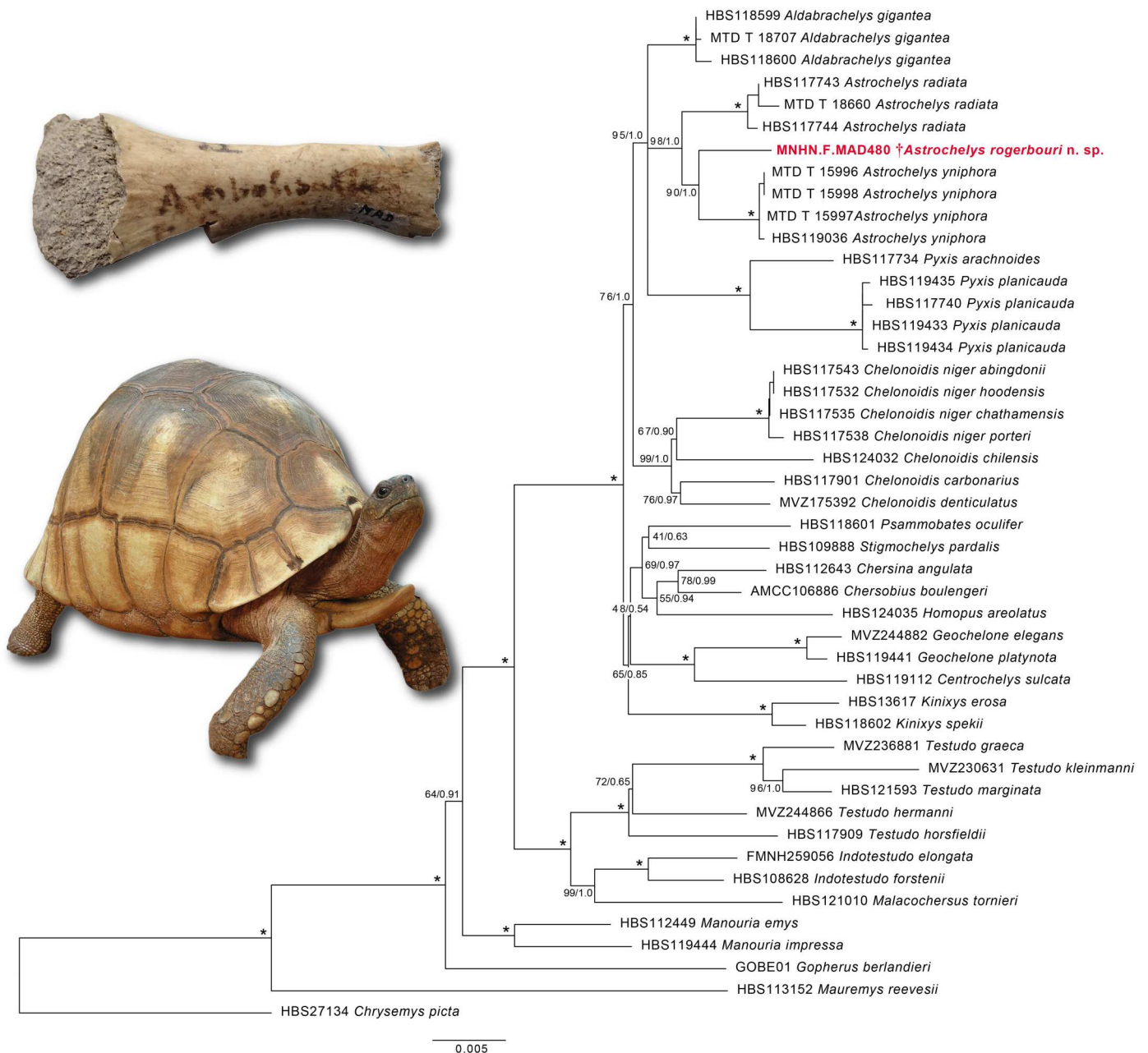


Fig. 3. Tortoise phylogeny based on nuclear DNA sequences, including an extinct Malagasy species (highlighted in red). The shown ML tree is based on four nuclear loci (*HMGB2*, *HNF1 α* , *R35*, and *TB73*; 3047 bp). Sample codes precede taxon names; for accession numbers, see table S3. Numbers at nodes are thorough bootstrap values and posterior probabilities from a Bayesian analysis yielding the same topology. Asterisks indicate maximum support under both approaches. Inset top: Holotype of *Astrochelys rogerbouri* n. sp. (photo: Uwe Fritz), bottom: *As. yniphora* (photo: Gerald Kuchling), the sister species of the extinct tortoise.

of *As. yniphora*, with a divergence resembling or exceeding the differences between other testudinid sister species (Fig. 3). This concordant mitonuclear divergence provides firm evidence that a third now-extinct *Astrochelys* species once roamed through Madagascar, in addition to the extant *As. radiata* and *As. yniphora*. The tibia of this previously unknown species was radiocarbon-dated to 1262 to 1069 cal yr B.P. (fig. S2).

Biogeography

Our molecular clock and ancestral range analyses (Fig. 2 and fig. S1, Supplementary Materials) support, in accordance with the phylogenetic branching patterns of the mitochondrial tree (Fig. 1), that *Cylindraspis* and the MAS clade independently dispersed out of Africa. The well-supported monophyly of the MAS clade implies, in line with our ancestral range analysis, that the pronounced diversity of Malagasy tortoises evolved on Madagascar, resulting in the small-bodied *Pyxis* species with shell lengths below 16 cm, the large-

bodied *Astrochelys* species with shell lengths up to 52 cm (8), and the extinct giant *Aldabrachelys* species reaching estimated shell lengths up to 135 cm (8, 12). Our genetic data derived both from mitochondrial and nuclear DNA also show that tortoise diversity on Madagascar was even greater than known before and that a third extinct *Astrochelys* species existed. This taxon is currently only known from a single tibia, which belonged to a tortoise of approximately 50 cm SCL (12). This size matches its extant congeners.

According to our molecular clock based on mitochondrial genomes, the diversification of Malagasy tortoises commenced in the Middle Oligocene (27.8 Ma ago), corresponding to the split of the giant *Aldabrachelys* lineage from a clade composed of the large-bodied *Astrochelys* and the small-bodied *Pyxis* lineages. *Astrochelys* and *Pyxis* separated slightly later at c. 25.1 Ma ago. Both genera diversified during the Miocene, with inferred mean divergence times ranging from 11.8 Ma ago for the two extant *Pyxis* species to 20.8 Ma ago for the divergence of *As. radiata* from the ancestor of *As. yniphora* and the previously unknown *Astrochelys* species.

Our ancestral range analysis (fig. S1) indicates that *Aldabrachelys* dispersed from Madagascar to the Granitic Seychelles and Aldabra, with the two Malagasy *Aldabrachelys* species being paraphyletic with respect to *Al. gigantea*. Little variation was detected among *Al. gigantea* sequences. The mitogenomes from Aldabra form a shallow clade together with subfossil material evidently (NHMUK PV R 3231) or putatively (NHMUK PV R 4682) from the Granitic Seychelles and many historical museum specimens that are also assumed to originate from the Granitic Seychelles. The only slightly divergent sample is NHMUK PV R 4682. This specimen was radiocarbon-dated to 1225 to 1063 cal yr B.P. (fig. S3), i.e., well before the 17th-century arrival of Europeans on the Seychelles (3). Dating NHMUK PV R 3231 failed, and resampling was not possible. Our ancestral range analyses (fig. S1) and paleogeographic evidence indicate that the Granitic Seychelles were the source for the overseas colonization of Aldabra (Fig. 4) and not vice versa and that the native tortoises on the Granitic Seychelles were exterminated and the islands restocked with Aldabra tortoises during the 19th century (see Discussion).

Systematic zoology

The genetic distinctness and the phylogenetic placement of MNHN.F.MAD480 (Figs. 1 to 3) indicate that it belongs to a previously unknown extinct *Astrochelys* species that survived at least until 1262 to 1069 cal yr B.P. (fig. S2). Despite the fragmentary material, we describe this species as new to science, on the basis of its deep genetic divergence.

Astrochelys rogerbouri n. sp.

Holotype

MNHN.F.MAD480, nearly complete tibia, Ambolisatra, Madagascar, Guillaume Grandidier, June 1900 (Fig. 3 and fig. S4). Ambolisatra is a well-known excavation site in southwestern Madagascar (12).

Diagnosis

A large-sized extinct tortoise species of the genus *Astrochelys* with an estimated SCL of 50 cm, only known from a single tibia. *Astrochelys rogerbouri* n. sp. can be separated from the two extant species of the genus by 59 diagnostic mutations in the mitochondrial genome (table S4 and data file S1) and 23 mutations in the reference alignment of four concatenated nuclear loci (*HMGB2*, *HNF1α*,

R35, and *TB73*; 3047 bp; table S5 and data file S2). The mitochondrial genome of the new species differs by an uncorrected *p* distance of approximately 8% from *As. radiata* and *As. yniphora* (data file S3); the concatenated nuclear loci of *As. rogerbouri* n. sp. differ by 0.9 to 1.3% from its two congeners (data file S4).

Etymology

The new species is named for the late Roger Bour (9 July 1947 – 7 March 2020), who was an outstanding herpetologist and expert on western Indian Ocean giant tortoises. Without his pioneering studies (3, 12, 14, 6) and his advice regarding museum specimens, this investigation would not have been possible.

Description of the holotype

A largely complete tibia, epiphyses are damaged (Fig. 3 and fig. S4). Maximum length of tibia is 62 mm, maximum width of proximal epiphysis is 26 mm, and maximum width of distal epiphysis is 15 mm. On the proximal epiphysis and shaft, the bone was sampled for sequencing and radiocarbon dating.

DISCUSSION

Our study contributes to a significantly better understanding of the biogeography and past diversity of western Indian Ocean tortoises. Our results require two distinct Eocene to Early Oligocene out-of-Africa dispersals of distantly related tortoises that resulted in independent radiations of Malagasy and Mascarene giant tortoises. We hypothesize that the extinct Mascarene *Cylindraspis* lineage spread from Africa in the Late Eocene to now-sunken islands of the Réunion hot spot (Saya de Malha, Nazareth, and Cargados Carajos banks) and diversified there before dispersing to the Mascarenes in the south (Fig. 4). There, further inter-island colonizations may have taken place resulting in four giant and one large-bodied tortoise species on Réunion (one species), Mauritius (two species), and Rodrigues (two species) (21). This scenario is compatible with our inferred divergence ages of the five Mascarene tortoise species (c. 27.5 to 4.5 Ma ago; Fig. 2) and the geological setting. The three modern-day islands, which are all volcanic, have emergence dates that vary from c. 10 Ma ago for Rodrigues (27) and c. 8.9 Ma ago for Mauritius (28) to c. 2.2 Ma ago for Réunion (29), supporting that at least some, if not all, *Cylindraspis* species did not diverge on the Mascarenes but arrived from the now-sunken northern islands. In contrast, the giant *Aldabrachelys* species evolved as part of a local tortoise radiation on Madagascar following the arrival of their African ancestor in the latest Eocene or Oligocene.

In our mitogenome-based scenario, both giant tortoise radiations commenced in the Middle Oligocene but led to a much higher body size diversity on Madagascar than on the Mascarenes (Fig. 1). The Malagasy radiation resulted in two giant tortoise species (*Aldabrachelys*), two small-bodied tortoise species (*Pyxis*), and three large-bodied tortoise species (*Astrochelys*), one of which was previously unknown. From Madagascar, the giant *Aldabrachelys* lineage dispersed much later (Pliocene) to the Granitic Seychelles and Aldabra (Fig. 4).

Disentangling diversity and biogeography of *Aldabrachelys* giant tortoises

Our study supports the notion that two extinct giant tortoise species lived on Madagascar, *Al. abrupta* and *Al. grandidieri*. These species have traditionally been recognized as distinct (8, 11, 12, 18). However, species-specific morphological differences mainly

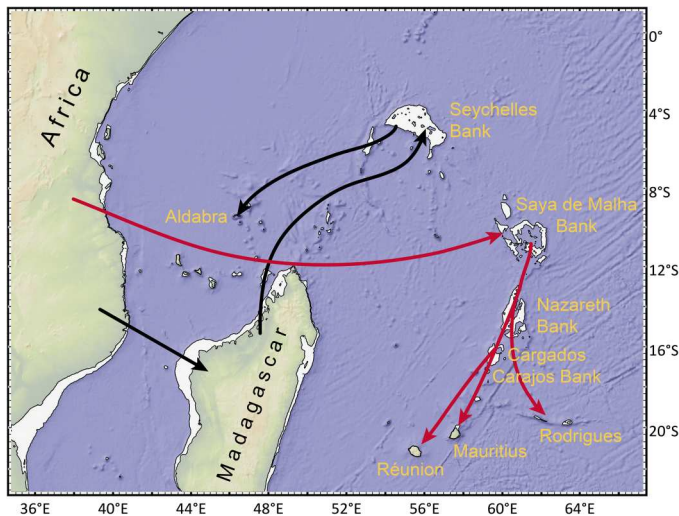


Fig. 4. Dispersal routes and radiation centers of western Indian Ocean giant tortoises. Black arrows: Malagasy tortoise radiation, including giant *Aldabrachelys*; red arrows: Mascarene *Cylindraspis* radiation. Note that the *Cylindraspis* lineage evolved and diversified on the now-sunken northern islands of the Réunion hot spot (now submersed Saya de Malha, Nazareth, and Carapados Carajós banks), while *Aldabrachelys* evolved on Madagascar and later dispersed to the Granitic Seychelles and subsequently to Aldabra. The map shows the key physiographical features of the western Indian Ocean. The base image was created using GeoMapApp (73). The ocean bed down to 120 m is shaded white and corresponds with the lowest lows in global sea level back to 630,000 years ago (74).

concern shell shape, and assigning specimens to one or the other species is difficult and often impossible. Therefore, recent studies (9, 30, 31) identified subfossil finds only as *Aldabrachelys*, without ascribing them to any species. Our genetic data, based on the historical type material, provide firm evidence that the two species were distinct and characterized by deeply divergent mitochondrial genomes. Contrary to earlier speculations (11), our data also show that extant Aldabra tortoises (*Al. gigantea*) are not conspecific with the extinct *Al. abrupta* from Madagascar, which represents their sister species of Early Pliocene age.

Besides Aldabra, we know from historical sources that native giant tortoises also occurred on the Granitic Seychelles and were heavily harvested for ship provision. The populations were largely eradicated by the early 19th century, when Aldabra came into the focus of exploitation (3). Currently, three subspecies of *Al. gigantea* are recognized from the Granitic Seychelles (8), although their exact historical distributions are unclear. *Aldabrachelys gigantea daudinii* is thought to be extinct, whereas *Al. g. arnoldi* and *Al. g. hololissa* are believed to survive in low numbers in captivity and introduced wild populations. The extant Aldabra population corresponds to the nominotypical subspecies *Al. g. gigantea* and has been translocated to many sites on the Granitic Seychelles and elsewhere (8, 10).

According to historical sources of the 18th and 19th centuries, large quantities of giant tortoises were shipped between different islands, not only within the Seychelles including Aldabra but also from the Granitic Seychelles and Aldabra south to the Mascarenes (3). This explains the unreliable and confusing locality data of all historical museum specimens that are presumed to originate from the Granitic Seychelles, including the type material of the three currently recognized subspecies. We found that the mitochondrial

genomes of all studied museum specimens presumed to come from the Granitic Seychelles are not differentiated from Aldabra tortoises.

If and when the specimens indeed originated from the Granitic Seychelles, then our sequence data imply either (i) that the native populations were not genetically differentiated from the giant tortoises on Aldabra or (ii) that the native populations were extirpated and by the early 19th century replaced by introduced Aldabra tortoises. Furthermore, the scientific descriptions of the currently recognized subspecies of *Al. gigantea* were based on one and the same genetic lineage, and the currently recognized subspecies designations should be abandoned.

The near-absence of sequence variation in the Aldabra-Seychelles clade (*Al. gigantea*) is notable (Fig. 1) and contrasts with the variation observed in other conspecific insular giant tortoises from the western Indian Ocean (*Cylindraspis* spp.) or elsewhere (Galápagos: *Chelonoidis niger* and Bahamas: *C. alburyorum*). That said, a single subfossil *Al. gigantea* (NHMUK PV R 4682) was slightly divergent. This bony shell was probably collected on the Granitic Seychelles during the Percy Sladen Trust Expedition in 1905 (11). Our AMS radiocarbon dating of this specimen (1225 to 1063 cal yr B.P.; fig. S3) revealed that the animal lived long before the discovery of the Seychelles in the early 17th century (3). If NHMUK PV R 4682 originated from the Granitic Seychelles, then this would support the idea that these islands once harbored a slightly distinct population that was driven to extinction. However, a second “subfossil” bone belongs to the remaining undifferentiated mitogenomes (NHMUK PV R 3231, a humerus from the Anse Royale marsh deposit, Mahé) (18). Unfortunately, this specimen could not be radiocarbon-dated, so that it remains speculative as to whether it is younger than the identification as subfossil suggests and represents an animal that was translocated during the time of intense tortoise harvesting.

If only the genetically divergent specimen is accepted as native to the Granitic Seychelles, then our ancestral range analyses support that *Al. gigantea* colonized Aldabra from the Granitic Seychelles (fig. S1), suggesting a dispersal route from Madagascar to the Granitic Seychelles and from there to Aldabra (Fig. 4). This is in line with the geological history of Aldabra, which was evidently at least once completely submerged during the last 200,000 years (32–35). Consequently, any giant tortoises that potentially occurred there before would have drowned. In contrast, the Granitic Seychelles, fragments of the ancient supercontinent Gondwana (36), were never completely flooded. This is evinced by ancient Gondwana relics such as the endemic sooglossid frogs or indotyphlid caecilians (37), and thus, the Granitic Seychelles facilitated the survival of giant tortoises that could later recolonize Aldabra after its reemergence. The genetic divergence of Aldabran *Al. gigantea* from their Malagasy sister species *Al. abrupta* (Figs. 1 and 2) precludes Madagascar as source area for the colonization of Aldabra, as speculated before (38).

In this context, it is noteworthy that probably no other group of large-sized vertebrates has such a pronounced propensity of overseas dispersal as large-bodied or giant tortoises (39). Extant or extinct species are known from many remote oceanic islands and archipelagos, including Aldabra, the Bahamas, Bermuda, the Canary Islands, Cape Verde, Fiji, Galápagos, the Granitic Seychelles, and Vanuatu (8, 10). For our study region, it was even described that a live Aldabra tortoise washed ashore in East Africa,

covered by barnacles (40). This provides primary evidence that large tortoises are capable of long-term survival when floating in the sea, making them excellently suited for long-distance overseas dispersal.

We hypothesize that the dynamic system of small islands north-east and east of Madagascar, including also now-submerged former islands of the Réunion hot spot (41), formed a dynamic system that hosted several populations of *Aldabrachelys* connected by regular overseas dispersal (39). Tortoise bones or early historical sightings identified as *Aldabrachelys* are known from many islands in the region (Fig. 5 and data file S5) (3, 18, 34, 38, 42), and at least some of these records unambiguously represent native occurrences. It remains, however, a mystery why no native tortoises have been recorded from the Comoros, although for other reptiles and even amphibians, numerous Late Cenozoic overseas dispersals have been inferred from Madagascar to this archipelago (43).

The unique diversity of the Malagasy tortoise fauna

In the course of our study, we found a previously unknown large-sized extinct tortoise species from southwestern Madagascar (*As. rogerbouri*). This brings the tally of recently extinct Malagasy tortoise species to three, so that three of seven endemic species (43%) vanished after the arrival of humans. The only known bone of *As. rogerbouri* was excavated at the same site (Ambolisatra) as the type material of *Al. abrupta*. This is most likely the reason why the type material of *As. rogerbouri* was originally assigned to a juvenile *Al. abrupta*; the tibia belonged to a tortoise of approximately 50 cm SCL (12). This size matches that of *As. yniphora*, the sister species of *As. rogerbouri*, for which a maximum SCL of 51.9 cm is known. In contrast, *As. radiata* reaches a maximum SCL of only 39.5 cm. These and all SCL values reported below are taken from the International Union for Conservation of Nature (IUCN) Checklist and

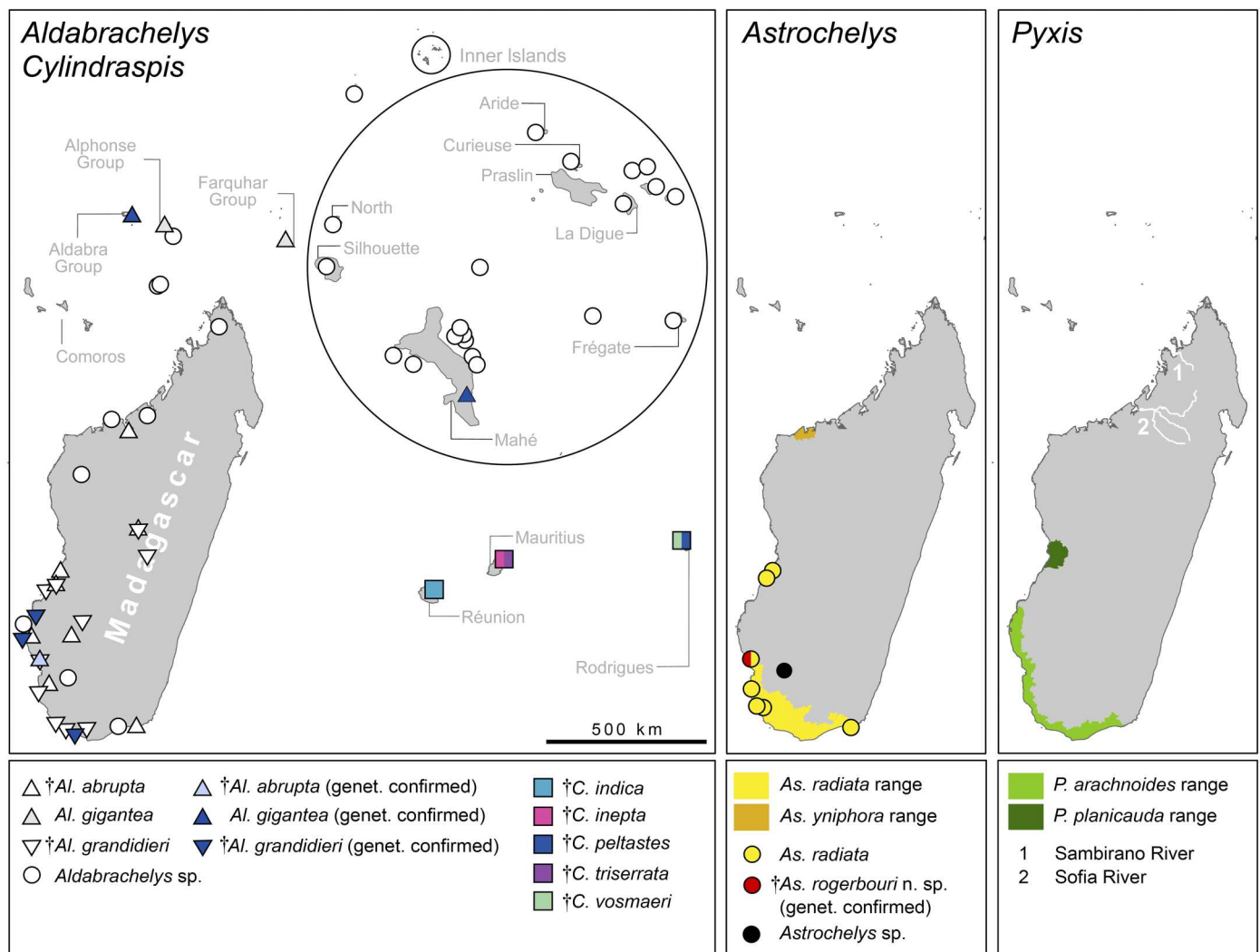


Fig. 5. Distribution of extant and extinct tortoise species on Madagascar and western Indian Ocean islands. Genus names of extinct taxa bear dagger symbols. If not otherwise indicated, genetically unconfirmed subfossil records are mapped (summarized from different sources; see data file S5). Divided or overlapping symbols indicate syntopic occurrences of the respective species. Distribution ranges for extant species are from the IUCN Checklist and Atlas of the Turtles of the World (8); introduced populations are disregarded. For the small Mascarene islands (Réunion, Mauritius, and Rodrigues), only one symbol per *Cylindraspis* species is shown; all species identifications are genetically supported (21). Native *Al. gigantea* are today restricted to Aldabra. The map for *Pyxis* spp. shows two rivers mentioned in the Discussion.

Atlas of the Turtles of the World (8). It is unknown when *As. rogerbourni* became extinct.

Among the material from Ambolisatra, the presence of both *As. rogerbourni* and *Al. abrupta* was confirmed by our mitochondrial genome data. This supports that at least one giant and one large-bodied tortoise species occurred there in microsympatry. However, it remains unclear whether additional tortoise species co-occurred at this site. Further material from Ambolisatra was morphologically attributed to the second extinct Malagasy giant tortoise, *Al. grandidieri* (8, 12), and to another *Astrochelys* species, the extant *As. radiata* (3), but these records lack genetic verification (Fig. 5). The previous misidentification of the holotype of *As. rogerbourni* as *Al. abrupta* exemplifies the difficulties in morphologically determining fragmentary tortoise bones. This makes us reluctant to accept the occurrence of *Al. grandidieri* and *As. radiata* at Ambolisatra without further confirmation. Notably, the bones from Ambolisatra assigned to *As. radiata* (3) could also represent *As. rogerbourni*, and the same is true for three other subfossil records of *As. radiata* and *Astrochelys* sp. outside the current distribution range of *As. radiata* (Fig. 5).

Similarly, the giant tortoise material from Ambolisatra identified as *Al. grandidieri* is very fragmentary (12), so that we regard its taxonomic assignment as questionable. However, the site of Ambolisatra is geographically within genetically confirmed records of *Al. grandidieri*. Furthermore, Ambolisatra is close to the northwestern limit of the current distribution range of *As. radiata*, giving some credibility to the possible former occurrence of *Al. grandidieri* and *As. radiata* there. Ambolisatra lies also within the range of the small-bodied tortoise species *P. arachnoides* (8). Thus, there is no doubt that five distinct tortoise species formerly occurred in southwestern Madagascar, one small-bodied species (*P. arachnoides*, maximum SCL: 15.7 cm), two large-bodied species (*As. radiata*, maximum SCL: 39.5 cm and *As. rogerbourni*, SCL: approximately 50 cm), and two giant species (*Al. abrupta*, maximum SCL: approximately 125 cm and *Al. grandidieri*, maximum SCL: approximately 135 cm), and some of them have lived microsympatrically. Such a high diversity of sympatric tortoise species is exceptional and exceeded only by South Africa, where up to seven species occur together or in close proximity (8). However, the diversity of the Malagasy tortoise radiation is unrivalled with respect to body size variation, as giant tortoises do not occur in South Africa.

Toward the north of Madagascar, the available data indicate a decrease in the past and present diversity of tortoise species (Fig. 5) (8, 12, 44). Both *Al. abrupta* and *Al. grandidieri* have been identified from sites in the central highlands where currently no tortoises occur (Fig. 5). *Aldabrachelys* records reach along the west coast to the very north of Madagascar. As far as morphologically identified, these northern remains have been attributed to *Al. abrupta*. Two extant native tortoise species live outside of southwestern Madagascar, and both are range restricted: In the Menabe region (central west coast), the small-bodied microendemic *P. planicauda* (maximum SCL: 16 cm) occurs, in a region from where the two extinct giant tortoise species were recorded (Fig. 5) (8). In the northwest, *As. yniphora* (maximum SCL: 52 cm), the extant sister species of *As. rogerbourni*, is currently confined to a small region (8) where it probably was sympatric with the extinct *Al. abrupta*. Therefore, according to current knowledge, the tortoise communities outside of southwestern Madagascar consisted of only two or three sympatric species.

Except for an introduced population of *Kinixys zombensis*, occurring in a small area of wet monsoon forest in northernmost Madagascar (8), no records of extant native tortoises are known from north of the Sofia River (Fig. 5) (8, 12), despite the existence of suitable dry habitat. However, the presence of extinct giant tortoises in the Ankarana massif in northern Madagascar has been reported (44), but apparently the smaller extant species have not been able to colonize the area. It is possible that the monsoon forest extending to the coast along the Sambirano River, which has been postulated as a barrier to gene flow in arid-adapted species (45), impeded the dispersal of the smaller species into the northern part of the island.

Our results open new perspectives for a better understanding of the natural history of the extinct and extant Malagasy tortoise species. It seems likely that each occupied a different niche. On the basis of stable carbon isotope data, it was initially suggested that *Al. abrupta* fed on a larger proportion of succulent plants than *Al. grandidieri*, which was proposed to be a grazer exploiting C₄ grasses (46). A much larger sample of giant tortoise remains later led to the conclusion that Malagasy giant tortoises primarily consumed C₃ plants (31), challenging the hypothesis that they were grazers with an ecological function akin to large herbivorous mammals (5, 7). However, among different sites, there was considerable variation in diet (31). Unfortunately, the underlying material was only identified as "giant tortoise," acknowledging species determination difficulties, so it remains unclear whether this variation in diet correlates with species-specific differences. If for stable isotope analyses bones would be used that have been taxonomically identified through aDNA, then it would open a new window for understanding the ecological niches of the extinct tortoises, which likely have played significant roles as plant consumers, seed dispersers, and germination enhancers. For instance, the decline of two tree species (*Sideroxylon sessiliflorum* and *S. grandiflorum*) on Mauritius could be related to the extinction of the two local *Cylindraspis* species. The giant tortoises are thought to have consumed the tree seeds and facilitated germination during gastrointestinal passage (47), and a similar scenario has been proposed for the Malagasy giant tortoises and the endemic baobab tree *Adansonia rubrostipa* (48).

Surprisingly, there are only few data directly comparing the dietary and habitat niches of Madagascar's extant tortoise species. The southwestern species *As. radiata* and *P. arachnoides* inhabit spiny thicket habitat, with the small-bodied *P. arachnoides* having a preference for coastal dunes and sandy substrates. The equally small-bodied *P. planicauda* lives in dry deciduous forest, while the highly endangered northwestern species *As. yniphora* until recently inhabited secondary growth habitat with bamboo, shrub thicket, and shrub palms. All four species are likely opportunistic feeders with grasses, leaves, flowers, and fruits having been recorded (49), but studies of possible differences in food preferences are lacking. The past sympatry of *As. radiata* and *As. rogerbourni* in southwestern Madagascar suggests that these two species had different habitat or dietary preferences, and it might be hypothesized that *As. yniphora* shared preferences with its extinct sister species *As. rogerbourni*. Testing niche differentiation and specialization of extant and extinct tortoises in Madagascar, e.g., using comprehensive analyses of stable isotopes, emerges as a promising field for future studies.

The discovery of *As. rogerbouri* underlines the power of aDNA approaches to reveal overlooked extinct species and to elucidate the past diversity of animal communities. Also sequencing subfossil material from tropical environments, long thought to be impossible or extremely difficult, becomes increasingly feasible, despite persisting challenges. We were able to generate near-complete mitochondrial genomes of 10 subfossil samples from Madagascar and the Seychelles and nuclear DNA sequences for one Malagasy sample, and some other recent studies using subfossil material from the tropics (23, 24, 50, 51) also yielded promising results. Besides analyzing molecular phylogenies and the discovery of new taxa, aDNA data from tropical environments can also be used to scrutinize ancient distribution patterns inferred from the morphology of subfossil material. Concerning Madagascar, our discovery suggests that other extinct tortoise species, and other large-bodied taxa, may be hidden among the extensive subfossil material in museum collections. Therefore, broad-scale sequencing of subfossil material would significantly enhance our understanding of the former diversity of the Grand Île.

MATERIALS AND METHODS

Studied specimens and wet lab protocols

Forty-three tortoise samples were studied in the aDNA facility of the Museum of Zoology, Senckenberg Dresden (tables S1 and S2). The samples were taken from 29 subfossil and 14 historical museum specimens. The latter tortoises were collected c. 300 to 200 years ago and preserved either as stuffed specimens, skeletons, or shells; two juvenile tortoises were ethanol-preserved. Depending on availability and DNA quality, one to three DNA extractions were performed for each sample, with 20 to 80 mg of dried tissue or bone powder per extraction. Tissue samples were processed using the DNeasy Blood & Tissue Kit (QIAGEN) and two final elution steps with 50 μ l of elution buffer. Bone powder was obtained by manually crushing small bone fragments in a porcelain mortar or by spot drilling several times at low speed for 2 to 3 mm into the cortical layer of larger bone parts using a Proxxon Micromot 50/E multitool equipped with 2- to 4-mm metal or stone drilling bits. Bone powder was processed according to a protocol optimized for the recovery of short DNA fragments (52). A maximum of 17 ng of DNA from the resulting lysates was converted into single-indexed, single-stranded Illumina sequencing libraries (53), including the removal of uracil residues by uracil-DNA glycosylase treatment. To increase the amount of endogenous DNA in the libraries, two rounds of in-solution hybridization capture (54) were performed in a dedicated capture-only workspace in the main laboratory, using mtDNA baits generated from long-range polymerase chain reaction (PCR) products of *Al. gigantea* (sample MTD T 18707), *As. radiata* (MTD T 18660), *P. arachnoides* (MTD T 18661), and *P. planicauda* (MTD T 1244) at an equimolar rate for *Aldabrachelys* and *Astrochelys* and long-range PCR products of *Centrochelys sulcata* (MTD T 16072) and *Chelonoidis chilensis* (MTD T 5657) at an equimolar rate for *Cylindraspis*. In addition, the seven most variable species-diagnostic nuclear loci (*AHR*, *HMGB2*, *HNFI α* , *NB22519*, *PAX1P1*, *R35*, and *TB73*) for the two extant *Astrochelys* species were selected from a recently published nuclear turtle phylogeny (22), each having a minimum uncorrected *p* distance of 0.7% between *As. radiata* and *As. yniphora*. Baits for these loci were generated from sequenced PCR products of *Al. gigantea* (MTD T

18707), *As. radiata* (MTD T 18660), and *As. yniphora* (MTD T 15996–15998). Long-range PCR, primer sequences, and PCR conditions are detailed in the Supplementary Materials (tables S6 to S8). All eight subfossil specimens from Madagascar and the two *Cylindraspis* specimens that yielded mitogenomes were processed for these nuclear loci. Sequencing was performed in-house on an Illumina MiSeq platform, generating 75-bp-long paired-end reads, and on an ABI 3730 Genetic Analyzer (Applied Biosystems). Sanger-sequenced data were manually checked for base calling errors using MEGA X (55).

Sequence assembly and sequence annotation

Raw next-generation sequencing sequence data were subjected to Skewer 0.2.2 (56) for adapter trimming and BBmap-suite 37.24 (57) for read merging (minimum length of 35 bp), quality filtering (minimum Q score of 20), and duplicate removal. For mitochondrial sequence assembly, the remaining quality-filtered reads (QFRs) were screened for contamination using FastQ Screen 0.11.4 (58) and a set of predefined mitogenomes (table S9). Any nontarget reads were excluded from the readpool (readpool 1). Then, all reads mapping to a taxonomically close reference genome were copied into a second readpool (readpool 2), and the mitogenomes were assembled with MITObim (59) and a two-step baiting and iterative mapping approach, with an allowed mismatch value of 2 and a starting seed according to table S1. For most samples, readpool 1 was used for the initial building of a preliminary genome and for the actual assembly. In some cases, readpool 2 was used for both steps or readpool 2 for the initial preliminary genome building and readpool 1 for the actual assembly (table S1). Resulting scaffolds were visualized and checked for assembly artifacts in Tablet (60). Artifacts were manually removed from the assembled contigs and all positions with coverage below threefold masked as ambiguous (*N*) using the maskfasta subcommand of BEDTools 2.29.2 (61). Sequence length distribution of mapped reads was calculated with a customized awk command and Microsoft Excel. The nuclear loci were assembled from the initial QFRs with MITObim in the same way, but using the following GenBank sequences or newly Sanger-sequenced data from fresh samples as starting seeds: MT958282 (*AHR*), MT965118 (*HMGB2*), MT959891 (*HNFI α*), MT960300 (*NB22519*), MTD T 15997 (*PAX1P1*), MT961199 (*R35*), and MTD T 15998 (*TB73*).

Phylogenetic, divergence time, and biogeographic analyses

Twenty-four mitogenomes were newly generated for this study, corresponding to eight subfossil specimens from Madagascar and one subfossil specimen evidently and another one putatively from the Granitic Seychelles plus 14 historical museum specimens. Data of the remaining 19 subfossil samples had to be excluded because of poor quality. The 24 mitogenomes were aligned with annotated mitogenomes from our previous studies (21, 23–25). After manually refining the preliminary alignment of 15,927 bp length (data file S6), each coding region was screened for internal stop codons using MEGA X. Challenging sequence features (stop codons, gene overlap, frameshifts, and spacer DNA) were removed. Our final alignment of 15,537 sites (data file S7) used for calculations comprised 98 sequences from all extant genera and species groups of tortoises (Testudinidae) plus two extinct tortoise lineages (five *Cylindraspis* species and *Chelonoidis alburysorum*). The two outgroup

taxa Geoemydidae (*Mauremys reevesii*) and Emydidae (*Chrysemys picta*) represent the successive sister taxa of the Testudinidae.

Only four nuclear loci could be sequenced in sufficient quality for a single subfossil specimen (MNHN.F.MAD480) from Madagascar. These data plus additional new sequences for *Al. gigantea*, *As. radiata*, and *As. yniphora* were concatenated and aligned with sequences of 33 testudinid taxa from a previous study (22) using the same outgroups as for mitogenomes. The final alignment (3047 bp; data file S2) for calculations contained 46 sequences from a similar taxon sampling as for the mitogenome dataset, albeit without *Cylindraspis* and the extinct *Chelonoidis* and Malagasy *Aldabrachelys* species.

Phylogenetic relationships were inferred both for the mitochondrial and nuclear data with the maximum likelihood (ML) and Bayesian inference (BI) approaches implemented in RAxML 8.0.0 (62) and MrBayes 3.2.6 (63). The best evolutionary models and partitioning schemes (tables S10 to S13) were assessed using PartitionFinder2 (64) with the greedy search algorithm and the Bayesian information criterion. For ML, the GTR + G substitution model was used for 10 independent searches with different starting conditions and the rapid bootstrap option. Finally, 1000 nonparametric thorough bootstrap replicates were calculated, and the values were plotted against the best tree. For BI, four parallel runs (each with eight chains) were conducted with two million generations (burn-in of 0.25, print frequency of 1000, and sample frequency of 500). Calculation parameters were examined using Tracer 1.7.1 (65). In addition, uncorrected *p* distances were calculated in MEGA X using the pairwise deletion option.

Divergence times for mitochondrial data were estimated using the uncorrelated lognormal relaxed clock models in BEAST 1.84 (66) using four well-established fossil calibration points (Fig. 2 and table S14). These calibration points were selected for a previous study involving paleontological expertise (20). They fulfill best-practice requirements for molecular clocks and were additionally cross-validated (table S15) (67).

On the basis of our mitochondrial dataset, the ML approach of BioGeoBEARS (68) served for inferring ancestral ranges. Our BioGeoBEARS analyses were based on extant distribution ranges corresponding to 12 biogeographic areas and tailored dispersal probabilities at seven time intervals from the Early Eocene to the Late Pleistocene-Holocene (data file S8) (21, 69). Details for molecular clock and ancestral range analyses are presented in the Supplementary Materials.

Radiocarbon dating

For two taxonomically and biogeographically important bone samples from Madagascar and presumably the Granitic Seychelles, purified collagen was radiocarbon-dated in the Leibniz Laboratory for Radiometric Dating and Stable Isotope Research, Christian Albrecht University, Kiel (Germany) using a HVE 3MV Tandemron 4130 system (70). The obtained conventional ¹⁴C ages were calibrated using the OxCal 4.4.2 software package (71) and the IntCal20 dataset for the northern hemisphere (72). Radiocarbon dating another sample from the Granitic Seychelles failed due to too low collagen content. Further details are explained in the Supplementary Materials.

Supplementary Materials

This PDF file includes:

Supplementary Materials and Methods
Figs. S1 to S6
Tables S1 to S16
References

Other Supplementary Material for this manuscript includes the following:

Data files S1 to S8

[View/request a protocol for this paper from Bio-protocol.](#)

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