Was there a second adaptive radiation of giant tortoises in the Indian Ocean? Using mitochondrial DNA to investigate speciation and biogeography of *Aldabrachelys* (Reptilia, Testudinidae)

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Abstract

A radiation of five species of giant tortoises (*Cylindraspis*) existed in the southwest Indian Ocean, on the Mascarene islands, and another (of *Aldabrachelys*) has been postulated on small islands north of Madagascar, from where at least eight nominal species have been named and up to five have been recently recognized. Of 37 specimens of Madagascan and small-island *Aldabrachelys* investigated by us, 23 yielded significant portions of a 428-base-pair (bp) fragment of mitochondrial (cytochrome *b* and tRNA-Glu), including type material of seven nominal species (*A. arnoldi*, *A. dussumieri*, *A. hololissa*, *A. daudinii*, *A. sumierei*, *A. ponderosa* and *A. gouffei*). These and nearly all the remaining specimens, including 15 additional captive individuals sequenced previously, show little variation. Thirty-three exhibit no differences and the remainder diverge by only 1–4 bp (0.23–0.93%). This contrasts with more widely accepted tortoise species which show much greater inter- and intraspecific differences. The non-Madagascan material examined may therefore only represent a single species and all specimens may come from Aldabra where the common haplotype is known to occur. The present study provides no evidence against the Madagascan origin for Aldabra tortoises suggested by a previous molecular phylogenetic analysis, the direction of marine currents and phylogeography of other reptiles in the area. Ancient mitochondrial DNA from the extinct subfossil *A. grandidieri* of Madagascar differs at 25 sites (5.8%) from all other *Aldabrachelys* samples examined here.

Keywords: Aldabra, *Aldabrachelys*, *Geochelone*, Indian Ocean, Madagascar, mtDNA, Seychelles

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Introduction

Large tortoises have colonized many oceanic islands by transmarine migration (Chevalier *et al*. 1935; Auffenberg 1974; Hutterer *et al*. 1998; Meylan & Sterrer 2000). Their buoyancy predisposes them to such journeys, as do the position of the lungs near the top of the shell, which makes them self-righting in water, and their long necks, which permit them to keep their heads well above the water surface and so breath easily. In historical times, three distinct groups of giant tortoises have occurred on oceanic

archipelagos: *Chelonoidis* in the Galápagos islands of the east Pacific ocean, *Aldabrachelys* on small islands north of Madagascar, including the Seychelles, and *Cylindraspis* in the Mascarene islands east of Madagascar. [These three units are either treated as independent genera, or as subgenera within the broader genus *Geochelone* s. lat, along with *Astrochelys*, *Geochelone* s. str and *Indotestudo*. *Aldabrachelys* has also been named *Dipsochelys*.] The first two survive but *Cylindraspis* became extinct in the early nineteenth century.

Such colonization of archipelagos may be followed by adaptive radiation. In the Galápagos, a single species, *Chelonoidis nigra*, has developed a number of distinctive island and intraisland races (Pritchard 1996; Caccone *et al*. 1999a,

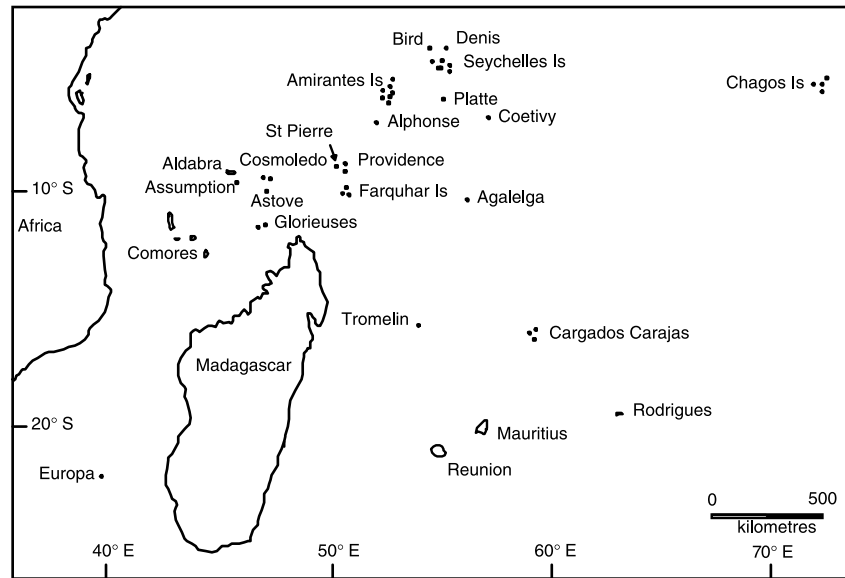Correspondence: E. N. Arnold. Fax: 0207 9425054, E-mail: ena@nhm.ac.uk

Fig. 1 Map of the southwest Indian Ocean showing islands, many of which once supported tortoise populations. Records of giant tortoises on islands north of Madagascar include the following: Comores — fossil (Bour 1982). Glorieuses — fossils in inclusions in a limestone matrix dated 125 000 years before present (BP; Battistini & Cremers 1972). Aldabra — fossils at two horizons: Bassin Cabri calcarinites, 500 000 years BP; Point Hodoul calcarinites, 100 000 years BP (Taylor *et al.* 1979); eye witness accounts from 1744 (Stoddart & Peake 1979). Assumption — fossil tortoise bones (Fryer 1911) and eggs (Honegger 1966); additional bones collected by S. Blackmore and M. Walker in 1977 have a radiocarbon date of 1140 ± 100 years BP (Burleigh & Arnold 1986). Astove — bones believed to be present (Fryer 1911); live animals seen in 1742 (Froberville 1848). Cosmoledo — fossil eggs (Fryer 1911). Denis — fossil eggs with a radiocarbon age of about 1308 ± 85 years BP (Burleigh 1979). Amirantes — fossil egg shells reported in conglomerate (Günther 1898), but no specimens now known. Granitic Seychelles — subfossil humerus from Anse Royale marsh deposit, Mahé (BMNH R3231), eyewitness accounts in 1609 by John Jourdain on Mahé and North Island near Silhouette (Stoddart & Peake 1979); many eyewitness reports in second half of the eighteenth century (Stoddart & Peake 1979). Unlikely or uncertain localities include (fide Stoddart & Peake 1979): African Banks, Agalega, Alphonse, Bird, Cargados Carajos, Chagos archipelago, Coetivy, Europa, Farquhar, Platte, Providence, St Pierre, Tromelin.

2002; Ciofi *et al.* 2002; Beheregaray *et al.* 2003) which show differences in the shape of the shell that may confer performance advantage in different habitats (Arnold 1979). *Cylindraspis* of the Mascarene islands radiated into no fewer than five species, four of which were sympatric with one of the others, and showed similar variation in shell shape to that present in the Galápagos (Arnold 1979; Austin & Arnold 2001; Austin *et al.* 2002). Similar radiation has been proposed for *Aldabrachelys* north of Madagascar, but there are conflicting opinions on this point.

Members of *Aldabrachelys* form a clade characterized by a derived condition involving a vertically elongate nasal opening of the skull. This is associated with complex nasal passages incorporating a valve-like structure that appears capable of isolating the olfactory area and may permit these tortoises to drink water through their nostrils, a possible adaptation to the dry climatic conditions in which they live, where available fresh water is often very shallow (Arnold 1979). Two sympatric species of *Aldabrachelys* were present in southwest and central Madagascar: *A. abrupta* and *A. grandidieri* (Vaillant 1885). Both are now extinct although they, respectively, occurred as recently as 750 ± 370 years and 1250 ± 50 years before present, and are

thus likely to have been exterminated by early human colonisers of Madagascar (Burleigh & Arnold 1986). Giant tortoise bones have also been found in northwest Madagascar at three localities close to Mahajanga (Bour 1992). These remains have previously been allocated to *A. abrupta* and, less certainly, *A. grandidieri* (details in Bour 1992) but they are fragmentary and their identity is at present uncertain.

When European mariners reached the area north of Madagascar in the seventeenth century, many of the small islands had populations of *Aldabrachelys* tortoises. Fossils with good stratigraphy, and radiocarbon dates for the more recent subfossil material, confirm that the presence of these tortoises on several islands predated the arrival of people (Fig. 1). Like their Madagascan congeners and the tortoises of the Mascarenes, most of the wild small-island *Aldabrachelys* populations have been exterminated, largely through overexploitation for food. They were taken as supplies by passing ships and exported to places where a tradition of eating tortoises had already developed, especially the Mascarene islands of Mauritius and Réunion (Stoddart & Peake 1979). Wild populations on the Seychelles were exterminated in this way by about 1800 and those on Astove, Assumption and elsewhere at approximately the

Table 1 Scientific names applied to non-Madagascan *Aldabrachelys* tortoises

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Testudo gigantea Schweigger 1812: 327. Type locality: 'Brasilien'. Type: lost.

Testudo dussumieri Gray 1831: 9. Type locality: Insula Aldebra. Type: RMNH 32311 (young animal in alcohol).

Testudo elephantina Duméril & Bibron 1835: 110. Type locality: Islands in Mozambique Channel (Anjouan, Aldabra, Comores) where frequently taken to Bourbon (Réunion) and Maurice (Mauritius). Lectotype: MHNP 7874.

Testudo daudinii Duméril & Bibron 1835: 123. Type locality: 'Indes orientales'. Type: MNHN 7640.

Testudo indica (non Schneider 1783).

Megalochelys indica Gray 1873: 724.

Testudo ponderosa Günther 1877: 35. Type locality: unknown. Type: BMNH 64.12.20.27 (skeleton and shell with scutes).

Testudo hololissa Günther 1877: 39. Type locality: Seychelles or Aldabra. Type BMNH 88.3.20.1 (stuffed).

Testudo sumeirei Sauzier 1892: 395. Type locality: Port Louis, Ile de France (= Mauritius), ex Seychelles. Type: BMNH 1947.3.4.1/1929.12.27.1. (stuffed).

Testudo gouffei Rothschild 1906: 753. Type locality: Thérèse Island, Seychelles. Type: BMNH 1949.1.4.66 (skeleton and shell; scutes and skin mounted separately).

Dipsochelys arnoldi Bour 1982: 121. Type locality: granitic Seychelles islands (the type specimen was actually brought from Réunion). Type: MNHN 9564 (paratypes in Paris and London).

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same time. The last remaining wild tortoises survive on Aldabra atoll. Not all animals taken from natural populations were killed, some being kept as pets or curios and allowed to breed. Such captive or feral animals or, more usually, their descendants exist on the Seychelles, Mauritius and Réunion in the Mascarenes, on Zanzibar, St Helena in the Atlantic and in zoological collections throughout the world. The frequent transport of non-Madagascan *Aldabrachelys* tortoises means that many animals in museum collections, which form the basis of systematic studies, lack firm original localities and, even when geographical origins have been assigned to them, these are often suspect. Only in a minority of preserved tortoises is there documentation that convincingly corroborates their provenance.

Many scientific names have been applied to non-Madagascan *Aldabrachelys* over the years (Table 1) and recent taxonomic treatments have sometimes been radically different. Several systematists have regarded all these animals as a single species and have applied the name *Geochelone gigantea* to it (for instance, Mertens & Wermuth 1955; Loveridge & Williams 1957; Wermuth & Mertens 1961; Arnold 1979). This course has been followed in many of the papers that have dealt with the ecology and other aspects of the surviving Aldabra tortoise population (for example Hnatiuk 1978; Bourn & Coe 1979; Coe *et al*. 1979; Stoddart & Peake 1979; Swingland & Coe 1979; Morgan & Bourn 1981; Hamilton & Coe 1982; Gibson & Phillipson

1983; Swingland 1983; Gibson & Hamilton 1984; Samour *et al*. 1987; Frazier 1988; Swingland *et al*. 1989; Hambler 1994; Rainbolt 1996; Bourn *et al*. 1999). An alternative view is that several species are represented, in which case *Aldabrachelys* would have undergone substantial radiation. At least eight species have been described and Bour (1984, 1985a,b) recognized five species of these and suggested localities for them: *A. daudinii*, *A. hololissa* and *A. arnoldi* (Granitic Seychelles), *A. sumierei* (Farquhar) and *A. elephantina* (Aldabra). In contrast, Gerlach & Canning (1998a,b) recognized four species: *A. hololissa* and *A. arnoldi* (Seychelles), *A. daudinii* (possibly Seychelles) and *A. dussumieri* (Aldabra). Not only are these species recognized by Gerlach and Canning but the authors believe that three (*A. hololissa*, *A. dussumieri* and *A. arnoldi*) still exist as captive specimens (Gerlach & Canning 1998b; Gerlach 1999) and they have commenced efforts to conserve these animals. The status of such forms is consequently of more than theoretical importance (see also Palkovacs *et al*. 2003).

Just as there are conflicting views about the taxonomy and possible radiation of non-Madagascan *Aldabrachelys*, so there are about the historical biogeography of the genus. Arnold (1979) suggested that *Aldabrachelys* was originally found on Madagascar and that Aldabra and nearby islands, and the Seychelles, were colonized from there by animals drifting on marine currents. This view receives some support from phylogenetic analyses of mitochondrial DNA (mtDNA) data from Madagascan and other tortoises (Palkovacs *et al*. 2002). Bour (1985a, 1992) also believed that *Aldabrachelys* reached the Seychelles from Madagascar but thought a propagule from the Seychelles then returned to Madagascar, later producing colonists that invaded Aldabra and neighbouring islands from there. Gerlach & Canning (1998a) proposed yet another hypothesis, that Aldabra and Madagascar were each independently colonized from the Seychelles.

In this paper, we assess the competing taxonomies of *Aldabrachelys* by examining a 428-base-pair (bp) segment of the mtDNA tRNA-Glu and cytochrome *b* genes from the types of the great majority of described forms. This enables us to assess the likelihood of a multispecies adaptive radiation and attempt to test the conflicting biogeographical hypotheses.

Material and methods

Thirty-seven individuals of *Aldabrachelys* were considered in this study (Table 2), including many old stuffed and skeletonized specimens in museum collections among which is type material of most nominal species. Animals certainly known to come from Aldabra (collected by A. Voeltzkow in 1893, and by D. Bourn in the 1990s) were included, as well as a number of living specimens believed by Gerlach & Canning (1998a,b) to represent three separate

Table 2 Origin of *Aldabrachelys* tortoise samples used in this study

Species	Specimen	Locality	Tissue source	DNA
Extinct madagascan species				
A. grandidieri	MNHN MAD3501, lectotype	Madagascar	subfossil bone	no
	BMNH 92.6.5.1	Madagascar	subfossil bone	yes
	BMNH R2019	Madagascar	subfossil bone	no
A. abrupta	MNHN MAD3500, lectotype	Madagascar	subfossil bone	no
	BMNH R2208	Madagascar	subfossil bone	no
	BMNH R5890	Madagascar	subfossil bone	no
	BMNH R11264	Madagascar	subfossil bone	no
Non-madagascan forms				
Type material				
A. arnoldi	BMNH 74.2.6.5, paratype	?	museum skin	yes
A. daudini	MNHN 7640, holotype	?	museum skin	yes
A. dussumieri	RMNH 3231, holotype	?	spirit preserved tissue	yes
A. elephantina	MNHN 7874, lectotype	Aldabra	museum skin	no
A. gouffei	BMNH 1949.1.4.66, holotype	?	museum bone	yes
A. hololissa	BMNH 88.3.20.1, syntype	Aldabra	museum skin	yes
A. ponderosa	BMNH 76.1.41/1947.3.4.94, cotype	?	museum bone	yes
A. sumeirei	BMNH 1929.12.27.1, holotype	Mauritius*	museum skin	yes
Material with definite locality				yes
A. gigantea	BMNH 1949.1.4.64	Aldabra	museum skin	yes
A. gigantea	D_Bourn	Aldabra	modern bone	yes
?	BMNH unregistered	Seychelles	swamp bone	no
?	BMNH R3231	Mahé	swamp bone	no
?	BMNH unregistered	Assumption	subfossil bone	no
Material with uncertain locality				
'A. arnoldi'†	NPTS_Hector	Seychelles*	blood, captive tortoise	yes
	NPTS_Bougainville 1	Seychelles*	blood, captive tortoise	yes
	NPTS_Bougainville 3	Seychelles*	blood, captive tortoise	yes
	AYO81789 — two individuals	Seychelles/zoos*	Palkovacs *et al.* (2002)	—
'A. daudini'	MNHN 11818	?	museum bone	yes
	MNHN 1942	'Seychelles'	spirit preserved tissue	no
'A. gigantea'	BMNH 1949.1.4.48	'Seychelles'	museum skin	yes
	BMNH 1949.1.4.48a	'Aldabra'	museum skin	yes
	BMNH 97.10.16.1	St Helena*	museum skin	no
	BMNH R9373	?	subfossil bone	no
'A. dussumieri'	NPTS_Silhouette Aldabra 1	Seychelles*	blood, captive tortoise	yes
	NPTS_Torti	Seychelles*	blood, captive tortoise	yes
	AYO811790 — eight individuals	Seychelles/zoos*	Palkovacs *et al.* (2002)	—
'A. hololissa'†	NPTS_Adam	Seychelles*	blood, captive tortoise	yes
	NPTS_Eve	Seychelles*	blood, captive tortoise	yes
	NPTS_Phoenix	Seychelles*	blood, captive tortoise	yes
	NPTS_Chiron	Seychelles*	blood, captive tortoise	yes
	AYO81791 — five individuals	Seychelles/zoos*	Palkovacs *et al.* (2002)	—
'A. sumeirei'‡	BMNH 1949.1.4.65	Mauritius*	museum skin	yes
	BMNH unregistered	?	museum skin	no

Specimen codes refer to museum registration numbers, named living tortoises or enBank Accession numbers. BMNH, The Natural History Museum, London; MNHN, Museum national d'Histoire naturelle, Paris; NPTS, Nature Preservation Trust of the Seychelles; RMNH, Nationaal Naturhistorisch Museum, Leiden (formerly Rijksmuseum van der Natuurlijke Historie).
*refers to location in captivity.
†fide J. Gerlach.
‡fide Rothschild.

extant species from the Seychelles and Aldabra. In addition, DNA sequences from 15 *Aldabrachelys* tortoises examined by Palkovacs *et al.* (2002) were obtained from GenBank (Accession numbers AY081789–91). Sub-fossil material from Mahé in the Seychelles and from Assump-tion island, and of both *A. abrupta* and *A. grandidieri* from Madagascar, was also investigated.

Samples consisted of dried skin from stuffed museum specimens, recent and subfossil bone, small pieces of ethanol-preserved tissue, and preserved blood taken from captive

animals. DNA extraction, polymerase chain reaction (PCR) amplification and automated DNA sequencing were carried out as previously described (Austin *et al.* 2002). Briefly, tissue samples were re-hydrated in 10 mM Tris–HCl (pH 8.0) and chopped finely using a sterile scalpel blade; bone samples were ground to a coarse powder in a presterilized coffee mill and decalcified in 0.5 M ethylenediaminetetraacetic acid (pH 8.0). Tissue, bone and blood samples were extracted using proteinase K digestion, phenol/chloroform extraction and centrifugal dialysis (Cooper 1994) or using a Qiamp Tissue Extraction kit (Qiagen) according to the manufacturer's instructions. A 428-bp fragment of the tRNA-Glu and cytochrome *b* genes was targeted using primary and secondary PCR amplifications of a set of four, 100–130-bp, overlapping fragments (museum and subfossil material) or as a single 428-bp fragment (recent material) using PCR conditions described by Austin *et al.* (2002). PCR primers were as follows (5'–3'; position of 3' nucleotide in complete human mtDNA sequence, Anderson *et al.* 1981): forward. TGA CTT GAA RAA CCA YCG TTG (14724, Palumbi 1996), ATC CAA CAT CTC AGC ATG ATG AAA (14841, Kocher *et al.* 1989), CAT CTC AGC ATG ATG AAA CTT CGG A (14848, Austin *et al.* 2002), ACT AGC ATT CTC ATC AGT AG (14946, Shaffer *et al.* 1997), TGC ATT TAC CTC CAY ATY GGC CG (15045, Shaffer *et al.* 1997); reverse. TGT AGG ATT AAG CAG ATG CCT AGT (14854, Austin *et al.* 2002), TCG GAT AAG TCA CCC GTA CTG (14966, Austin *et al.* 2002), AAG TCA TCC GTA TTG TAC GTC TCG (14957, Austin *et al.* 2002), GGT AAG AGC CGT ART AAA GTC (15048, Austin *et al.* 2002), CCC TCA GAA TGA TAT TTG TCC TCA (15149, Palumbi 1996), TCA GAA TGA TAT TTG TCC CCA TGG T (15145, Austin *et al.* 2002). PCR products were gel purified (Boyle & Lew 1995) and sequenced directly using an ABI 373 or 373 DNA Sequencer according to the manufacturer's instructions. Processing of all museum and subfossil material followed strict procedures appropriate for ancient DNA designed to minimize the possibility of contamination (Austin *et al.* 1997a,b, 2002) and included a separate, dedicated ancient DNA laboratory, negative extraction and PCR controls, and repeated extraction, PCR and sequencing on museum and subfossil specimens.

For comparison, homologous DNA sequences from a wide range of testudinids outside *Aldabrachelys* were obtained from GenBank and the literature. These include the following (letters and figures are GenBank accession numbers; species of *Indotestudo*, *Manouria*, *Chelonoidis* and *Astrochelys* are listed in GenBank as members of *Geochelone*): Africa — *Geochelone pardalis* AF371238, *G. sulcata* (Momont 1998); India — *G. elegans* AF371237; Southeast Asia — *Indotestudo elongata* AF371235, *I. forsteni* (Momont 1998), *Manouria emys* (Momont 1998); the Galápagos islands — *Chelonoidis nigra* AF0200899; South America — *C.*

denticulata AF192941, *C. carbonaria* AF192928 and *C. chilensis* AF192929; Madagascar, all living endemic tortoises (Caccone *et al.* 1999b) — *Astrochelys radiata* AF371239, *A. yniphora* AF020896, *Pyxis arachnoides* AF020894, *P. planicauda* AF020895; the Mascarene islands, all endemic tortoises (Austin & Arnold 2001) — *Cylindraspis indica* AF371243, *C. triserrata* AF371248, *C. inepta* AF371250, *C. peltastes* AF371253 and *C. vosmaeri* AF371258.

Results

DNA sequence was obtained from 23 of the 37 *Aldabrachelys* samples investigated (Table 2). Twenty-two of the 26 nonfossil samples (skin, recent bone, spirit-preserved tissue and blood) yielded DNA but only one of 11 subfossil bones processed did so. No DNA was recoverable from the four specimens of *A. abrupta*, but sequences were obtained from one *A. grandidieri*, from type material of seven nominal species (but not that of *A. elephantina*), from two specimens definitely known to come from Aldabra, and from 13 specimens of uncertain provenance. Full-length, 428-bp sequences are available for 18 specimens and partial sequences for the following 20, the number of base pairs obtained being given in parentheses: *A. arnoldi*, paratype — BMNH 74.2.6.6 (336 bp), *A. dussumieri*, holotype — RMNH 3231 (336 bp), Aldabra — BMNH 1949.1.4.64 (102 bp), material of uncertain origin — NPTS_Phoenix (398 bp), NPTS_Chiron (397 bp), 15 sequences from Palkovacs *et al.* (2002) (386 bp)

In all, 37 sequences for non-Madagascan *Aldabrachelys* tortoises are available, when the 15 samples of Palkovacs *et al.* (2002) are included. Variation among the sequences is minimal (Fig. 2). Thirty-three samples share an identical haplotype (haplotype A) previously reported by Palkovacs *et al.* (2002), while the remaining four individuals represent four haplotypes that diverge from haplotype A by one, two or four nucleotide substitutions (0.23–0.92% divergence). The pattern of nucleotide variation is typical for mtDNA with five transition substitutions (all in the cytochrome *b* gene), two transversions (one in the tRNA-Glu and two in the cytcochrome *b* gene) and one deletion (in the tRNA-Glu gene). Relationships between the five haplotypes are shown in Fig. 2(b). The common haplotype A is central with the remaining haplotypes branching off from this. In contrast to the lack of diversity within non-Madagascan samples, their most common haplotype (A) differs from that of the extinct Madagascan *A. grandidieri* by 25 nucleotide substitutions (5.8% divergence), confirming the distinctiveness of this species, and by 31–48 nucleotide substitutions (8–11% divergence) from all other testudinid sequences included in this study. The *A. grandidieri* sequence and the common *A. gigantea* sequence have been deposited in GenBank (Accession Numbers: AF371240 and AF371241).

(a)

| Sample Code	111223 11368295 06525593	Haplotype
A. gigantea consensus	AACGGACC	A
BMNH 74.2.6.5	..????..	A
MNHN 7640	..??.?..	A
MNHN 3231	..????AT	B
BMNH 1949.1.4.66	....	A
BMNH 88.3.20.1	....	A
BMNH 76.1.41	..????..	A
BMNH 1929.12.27.1	....	A
BMNH 1949.1.4.64	??????.?	A
D_Bourn	....	A
NPTS_Hector	....	A
NPTS_Bougainville 1	?.......	A
NPTS_Bougainville 3	C.......	D
AYO81789	??......	A
MNHN 11818	....	A
BMNH 1949.1.4.48	....	A
BMNH 1949.1.4.48a	..TAAG..	C
NPTS_Silhouette Aldabra 1	....	A
NPTS_Torti	?.......	A
AYO81790	??......	A
NPTS_Adam	.-......	E
NPTS_Eve	....	A
NPTS_Phoenix	??......	A
NPTS_Chiron	??......	A
AYO81791	??......	A
BMNH 1949.1.4.65	....	A

(b)
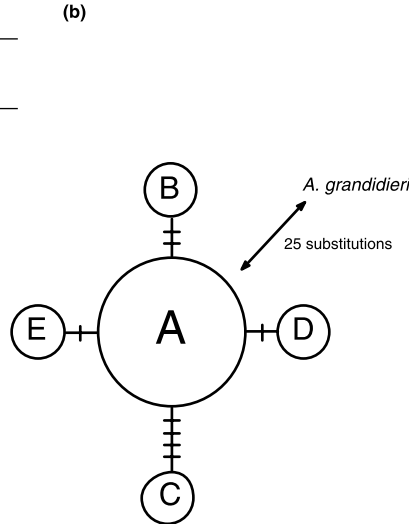
Fig. 2 (a) Nucleotide variation at eight variable sites among 428 bp of mtDNA sequence for 37 individual tortoises representing seven nominal species of *Aldabrachelys*. Individuals are listed in the same order as they appear in Table 2. Dots indicate identity with the most common haplotype (haplotype A). Numbers refer to the nuclotide position along the 428 bp of sequence. (b) Haplotype network describing relationships between the five haplotypes found in the same 37 *Aldabrachelys* tortoises and the sequence from *A. grandidieri*. Hatches across lines joining haplotypes each represent a single nucleotide substitution and circle size is proportional to haplotype frequency.

Discussion

The uniformity in cytochrome *b* sequence in non-Madagascan *Aldabrachelys* demonstrated here extends previous findings of uniformity in mtDNA from living tortoises in captivity but of uncertain original locality (Palkovacs *et al.* 2002). Subsets of 32 living specimens of tortoise recently allocated to three species *A. arnoldi*, *A. dussumieri* and *A. hololissa* showed no variation in over 13 000 bp of mtDNA sequence from three genes (cytochrome *b*, 16SrRNA and 12SrRNA) including a shorter 386-bp fragment of the cytochrome *b* gene analysed here (Palkovacs *et al.* 2002). An expanded study of 55 from both captive and wild populations found no variation in mtDNA control region sequences and low variability at eight microsatellite loci (Palkovacs *et al.* 2003). The conflicting hypotheses about the systematics and radiation of non-Madagascan *Aldabrachelys* can therefore be considered in the light of quite extensive information about molecular variation in both living and historical populations of non-Madagascan *Aldabrachelys*.

The case for a single-species interpretation of studied material

The lack of any differences within the cytochrome *b* sequence of six of the seven types of nominal species contrasts strongly with the situation among tortoise species that are widely accepted as being valid. These show marked differences in their cytochrome *b* sequence. Among the five Mascarene species of *Cylindraspis*, differences range from 11 to 72 bp (2.5–17%) (Austin & Arnold 2001). In *Pyxis* the two species, *P. arachnoides* and *P.*

planicauda differ by 31–34 bp in a 386-bp fragment of cytochrome *b* (8.0–8.8%) and in *Astrochelys*, *A. radiata* and *A. yniphora* differ by 32–34 bp (8.2–8.8%) (Caccone *et al.* 1999b). Substantial differentiation also occurs in the other species listed elsewhere (Caccone *et al.* 1999b). Accepted species of other heterotherm amniotes, such as lizards, frequently show similar large differences in their mitochondrial genes, for instance 3.2–9.1% in cytochrome *b* and 12S rRNA of species of *Tarentola* geckos (Carranza *et al.* 2000), and 5.2–11.6% in *Gallotia* lacertids (S. Carranza and E. N. Arnold, personal observation).

If the six types with uniform cytochrome *b* were regarded as probably belonging to a single species, this would also include the 11 additional animals that show no differences from them. The individuals within the studied sample that differ from the common haplotype by 1–4 bp (0.23–0.9% divergence), including the type of *A. dussumieri* would also be referable to the same single species, for even closely related tortoise species that are widely accepted show much greater differentiation (see above). Indeed accepted species often exhibit more difference among their members than that present in the 37 non-Madagascan *Aldabrachelys* considered here. For instance, in the same 428-bp fragment of cytochrome *b*, differences of 2, 3 and 5 bp were encountered within *Cylindraspis inepta*, *C. vosmaeri* and *C. indica*, respectively, even though sample size was low (three, four and five individuals) (Austin & Arnold 2001). Within the Galápagos species, *Chelonoidis nigra*, differences of about 10 bp were encountered in 386 bp of cytochrome *b* and 568 bp of 16S (Caccone *et al.* 1999a). Some or all of the deviant haplotypes could conceivably represent conspecific populations from other islands or archipelagos north of Madagascar. However, the

range of divergence encountered could just as easily occur within a single island as it does in *Cylindraspis*, where all species are each confined to one island. Again, a sample of 11 *Chelonoidis nigra* from Santa Cruz island in the Galápagos archipelago includes six haplotypes of 416 bp of cytochrome *b* varying at a total of six sites. As in the non-Madagascan *Aldabrachelys* studied here, the rarer haplotypes differ from the commonest one by 1–4 bp (Caccone *et al.* 2002).

Although the sequence presented here suggests a single species, it does show more variation than the uniform cytochrome *b* sequence reported by Palkovacs *et al.* (2002) in living *Aldabrachelys*. The greater variation in our sample may partly be the result of PCR artefacts caused by the age and poor condition of many of the specimens used (Hofreiter *et al.* 2002). Again, there may have been some sampling of extinct lineages.

If available specimens of non-Madagascan *Aldabrachelys* were regarded as belonging to a single species, the undoubted morphological variation present would have to be interpreted in this context. Variation in shell shape does not necessarily indicate the presence of more than one species. For instance it is very marked in the Galápagos tortoise, *Chelonoidis nigra*, which is generally regarded as a single species (Pritchard 1996; Caccone *et al.* 1999a, 2002), and here substantial morphological variation can occur within a single island (Pritchard 1996; Caccone *et al.* 1999a). Furthermore tortoise shells are, notoriously, phenotypically plastic and conditions in captivity, such as diet, physical environment, conditions of incubation and disease can produce substantial, sometimes pathological, modifications. In some chelonians such as *Apalone ferox* (Trionychidae) skulls are also quite labile (Dalrymple 1977). Phenotypic effects may well have modified some of the *Aldabrachelys* specimens that have reached museums, or have been studied when alive, as these were often held in captivity beforehand and sometimes were bred there. Such lability seems counterintuitive: tortoise shells are such robust and impressive structures that it is easy to believe they nearly always carry a significant phylogenetic signal but this is not the case. Assessing the significance of differences in shell shape is especially difficult in *Aldabrachelys* because no careful assessment of variation in this feature has been made in any natural small-island population. This is true even for the surviving Aldabra tortoises, which have been intensively studied in other ways, although there do appear to be substantial differences between individuals (J. Frazier, personal communication).

Geographical origin of studied non-Madagascan Aldabrachelys *under a single-species interpretation*

The geographical origin of the *Aldabrachelys* type specimens studied here, and the remaining animals with little or no differences from them in cytochrome *b* and with equally poor locality data, may well be Aldabra atoll. Most of these tortoises have haplotypes identical with a specimen certainly known to come from there (collected by D. Bourn; the partial sequence from that collected by A. Voeltzkow, BMNH 1949.1.4.64, also showed no differences). Aldabra is also a likely origin of all or most other available material of non-Madagascan *Aldabrachelys* because it has been the main source of wild giant tortoises in the Indian Ocean since the 1820s or earlier. Animals were being shipped from Aldabra to Mauritius and to Mahé in the Seychelles in 1822 (Moresby 1842) and by 1839 there was regular export to Mahé (Harrison 1839). In the 1830s numerous newspaper advertisements appeared in Réunion for newly landed Aldabra tortoises (Bour 1980, 1981) and in 1842 two ships collected 1200 tortoises from the atoll (Kersten 1871). Removal of tortoises from the island continued at least intermittently until the 1970s (S. Blackmore, personal communication). As none of the nominal species of which type material was investigated here were described before 1835, an Aldabran origin for them is quite possible. If all the animals in the studied sample actually came from Aldabra, the mtDNA of the endemic Seychelles tortoises would remain effectively unsampled and none of the nominal species investigated here could have come from that archipelago. Alternatively, the minor differences in cytochrome *b* encountered could be the result of some specimens coming from elsewhere.

Biogeography of Aldabrachelys *under a single-species interpretation*

Arguments that *Aldabrachelys* colonized Madagascar from the Seychelles (Bour 1985a, 1992; Gerlach & Canning 1998a) are based on the topology of phylogenetic hypotheses derived from interpretations of morphology. Because the topologies include two or more putative Seychelles taxa that form successive basal branches (in contrast to the two extinct Madagascan species which form a more terminal sister pair), it is most parsimonious to assume a Seychelles origin. The present molecular work cannot throw light on this hypothesis as only one taxon is discernible on the islands north of Madagascar and that may come from Aldabra rather than the Seychelles. Nor, with only a single Madagascan taxon (*A. grandidieri*) sampled can a Madagascan origin of Aldabra tortoises be tested on the basis of the mtDNA obtained here. As noted, there is some molecular evidence that *Aldabrachelys* colonized the Seychelles and Aldabra from Madagascar (Palkovacs *et al.* 2002). To those can be added evidence from the direction of marine currents and the phylogeography of more speciose reptile groups in the area. Both these sources of inference support a Madagascan origin for tortoises with later spread to more northern

islands. The source area is likely to be in the north of Madagascar, where imprecisely identified *Aldabrachelys* remains occur. Currents flow in the appropriate direction for colonization of Aldabra and the Seychelles (Taylor *et al*. 1979) and other faunal elements, especially lizards, suggest a Madagascan source for taxa occurring on these smaller islands. Phylogeny based on DNA shows the chameleon, *Calumma tigris*, of the Seychelles clearly has Madagascan origins (Raxworthy *et al*. 2002) and the same is true of the *Phelsuma* geckos of the Seychelles and Aldabra (Radtkey 1994; Austin *et al*. submitted for publication). Apart from extant *Phelsuma* and *Aldabrachelys*, the living and extinct Pleistocene reptile fauna of Aldabra totals nine species (Arnold 1976). Eight of these have their closest relatives in Madagascar and in five likely paraphyly in that area indicates that it was the source region. The forms concerned are the iguanid *Oplurus* cf. *cuvieri*, the geckos *Paradura* cf. *stumpfii* and *Geckolepis* cf. *maculata*, and the skink *Amphiglossus* cf. *valhallae*. There appear to be no overt cases where lizards have migrated in the opposite direction, from the Seychelles or Aldabra to Madagascar.

The case for an alternative, multispecies interpretation of studied material

A different interpretation of the morphological variation present in non-Madagascan *Aldabrachelys* is that it really does reflect the presence of several species, including species from the Seychelles (Bour 1984; 1985a,b; Gerlach & Canning 1998a,b). If this were true, the lack of significant differentiation in the cytochrome *b* sequence would be explainable if speciation and morphological evolution had occurred relatively recently, so there was not sufficient time for substantial changes in this particular gene fragment. Although no cases of this sort are known in other tortoises, there are examples in other taxonomic groups, where morphologically distinct sympatric species have evolved without marked changes in the mitochondrial genome, for instance in the cichlid fish of East African lakes (Rossiter 1995).

Conclusions

On the basis of its uniformity compared with other tortoises, the mtDNA of non-Madagascan *Aldabrachelys* studied here suggests that only a single species may be involved. Although type material of several nominal species is included in the sample, all of it may come from Aldabra. This interpretation contrasts with one postulating several species resulting from relatively rapid speciation and evolution, some of which are thought to come from the Seychelles. Future investigations may elucidate this situation. It would be possible to look for material certainly

from the Seychelles, such as subfossils from sites on those islands which might also yield DNA. Control region and microsatellites of the type specimens already investigated for cytochrome *b* could be studied and more old preserved specimens in museums could be screened to see if very different haplotypes exist that may represent non-Aldabran material. More specimens from Aldabra itself could be checked to see if the slightly deviant haplotypes already encountered in this study actually occur there. At the same time the morphological variation used to support the multispecies interpretation needs to be assessed in the light of likely phenotypic variation. Anecdotal information on the effects of diet, environment and incubation conditions needs to be investigated more systematically, to see if these factors alone could account for the morphological variation encountered.

Acknowledgements

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