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The evolutionary origin of Indian Ocean tortoises (*Dipsochelys*)

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Abstract

Today, the only surviving wild population of giant tortoises in the Indian Ocean occurs on the island of Aldabra. However, giant tortoises once inhabited islands throughout the western Indian Ocean. Madagascar, Africa, and India have all been suggested as possible sources of colonization for these islands. To address the origin of Indian Ocean tortoises (*Dipsochelys*, formerly *Geochelone gigantea*), we sequenced the 12S, 16S, and cyt *b* genes of the mitochondrial DNA. Our phylogenetic analysis shows *Dipsochelys* to be embedded within the Malagasy lineage, providing evidence that Indian Ocean giant tortoises are derived from a common Malagasy ancestor. This result points to Madagascar as the source of colonization for western Indian Ocean islands by giant tortoises. Tortoises are known to survive long oceanic voyages by floating with ocean currents, and thus, currents flowing northward towards the Aldabra archipelago from the east coast of Madagascar would have provided means for the colonization of western Indian Ocean islands. Additionally, we found an accelerated rate of sequence evolution in the two Malagasy *Pyxis* species examined. This finding supports previous theories that shorter generation time and smaller body size are related to an increase in mitochondrial DNA substitution rate in vertebrates.

Keywords: *Dipsochelys*; Giant tortoises; Aldabra; *Pyxis*; Island colonization; Mitochondrial DNA; Rate variation

1. Introduction

Fossil evidence shows that giant tortoises once existed on every continent, except Antarctica and Australia (Auffenberg, 1974). In recent times, however, giant tortoises have survived only on oceanic islands, including the Galápagos Archipelago in the Pacific Ocean and a host of islands in the western Indian Ocean, including Aldabra, the Comoros, and the granitic Seychelles (Fig. 1). Beginning in the early 1600 s, Indian Ocean tortoises (*Dipsochelys*) were exterminated from the majority of these islands and moved between islands in great numbers by early European settlers (Stoddardt et al., 1979). Therefore, reconstructing their taxonomy and biogeography has been challenging. Fossil remains have been found on Madagascar, the Comoros (Bour, 1994), Assumption, Astove, Cosmoledo, Denis, and Glorioso (Stoddardt et al., 1979). Historical records, although often unreliable and difficult to interpret, show that giant tortoises have recently occupied Mauritius, Réunion, Rodrigues, and the Seychelles (Stoddardt et al., 1979). By 1840, however, the only remaining wild tortoise population in the western Indian Ocean resided on the island of Aldabra.

Indian Ocean tortoises are second only to Galápagos tortoises (*Geochelone nigra*) in maximum size, with adults reaching a weight of 120 kg and a carapace length of 105 cm (Ernst and Barbour, 1989). A distinguishing morphological characteristic of *Dipsochelys* is the modification of the nasal passages to allow water to be drawn up through the nostrils (Arnold, 1979; Bour, 1982; Gerlach and Canning, 1998). This peculiar nasal arrangement is thought to be an adaptation to the dry climate of oceanic islands, allowing the tortoises to drink from very shallow pools of water (Frazier, 1971; Swingland in Arnold, 1979; Bour, 1984a).

The taxonomy of Indian Ocean giant tortoises has been revised multiple times. Schweigger (1812) first described the Indian Ocean tortoises as a single species,

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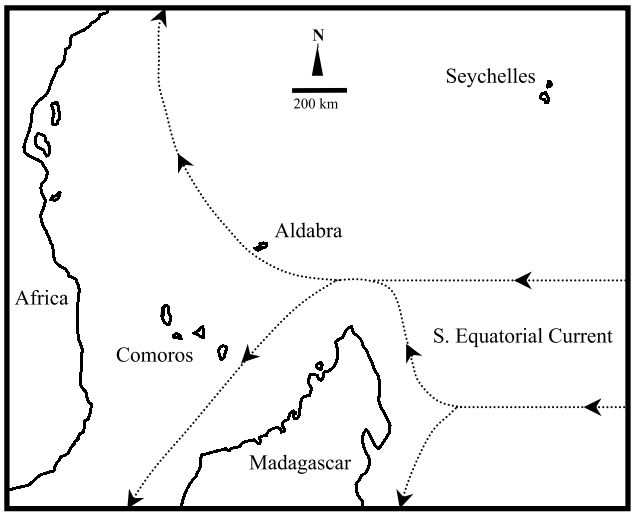

Fig. 1. Map showing the west Indian Ocean islands of Madagascar, the Comoros, Aldabra, and the Seychelles. The South Equatorial Current flows west until it hits the coast of Madagascar, producing currents that flow north around the tip of Madagascar towards Aldabra and the Comoros (Tomczak and Godfrey, 1994). These currents likely transported tortoises from Madagascar to oceanic islands.

Testudo gigantea. Rothchild (1915) recognized a range of rather poorly defined species. Arnold (1979) defined the Aldabra–Seychelles giant tortoises as a single variable species (*Geochelone gigantea*) in the subgenus *Aldabrachelys* and five extinct species from the Mascarene Islands in the subgenus *Cylindraspis*. Bour (1982, 1984b) suggested *Dipsochelys* as a replacement for *Aldabrachelys* and raised *Dipsochelys* to generic status. Since the species name *gigantea* appears to have been wrongly assigned to an Aldabran tortoise—the lost holotype examined by Schweigger in 1812 is now thought to have been either a Mascarene or South American tortoise—it was not reassigned to any Indian Ocean tortoises (Bour, 1984b; Gerlach and Canning, 1998; Pritchard, 1986).

Recently, Gerlach and Canning (1998) have recognized and redescribed six species of Indian Ocean giant tortoises within the genus *Dipsochelys*. An extinct species presumably of Seychelles origin, *D. daudinii* is known from only two museum specimens, the holotype (lacking collection data) and a juvenile (Gerlach and Canning, 1998). The two extinct Malagasy species (*D. abrupta* and *D. grandidieri*) are known from fossil remains found across Madagascar. These remains have been dated back to about 2000 years before the present (Arnold, 1979). Of the three extant species, two are possibly of Seychelles origin (*D. arnoldi* and *D. hololissa*) and now exist only in captivity. The Aldabran tortoise (*D. dussumieri*) is the only species currently living in the wild. Bourne and Coe (1978) estimated that 150,000 individuals survived on Aldabra, although resource depletion (Gibson and Hamilton, 1984) and competition with goats (Hambler, 1984) have since caused population declines. Aldabran tortoises were recently introduced to Curieuse Island (1978–1982) and Fregate Island (1968–1973) in the Seychelles as a conservation strategy and tourist attraction (Hambler, 1994).

The evolution and pattern of island colonization of *Dipsochelys* have remained a topic of much debate. Arnold (1979) suggested that Aldabra and its surrounding islands were colonized from Madagascar. Gerlach and Canning (1996) proposed that the Comoros might have served as the source of colonization for Aldabra. An alternative, proposed by Bour (1984b) and supported by a phylogeny based on osteological characters (Gerlach and Canning, 1998), suggests that Madagascar and Aldabra were colonized from the Seychelles.

In this study, we address the evolutionary origin of *Dipsochelys* by reconstructing a phylogeny of Malagasy, African, Indian, and Aldabra–Seychelles tortoises using mitochondrial DNA (mtDNA) sequences from the 12S and 16S ribosomal RNA (rRNA) and cyt *b* genes. In our analysis, we have included all *Geochelone* species endemic to Africa (*G. sulcata* and *G. pardalis*) and India (*G. elegans*), all tortoises endemic to Madagascar (*Geochelone radiata*, *Geochelone yniphora*, *Pyxis arachnoides* and *Pyxis planicauda*), and the three extant *Dipsochelys* species (*D. dussumieri*, *D. hololissa* and *D. arnoldi*). As outgroups, we have used the North American gopher tortoise (*Gopherus polyphemus*) and the Galápagos tortoise (*G. nigra*).

2. Materials and methods

2.1. Materials

A total of 32 *Dipsochelys* individuals were used in our analysis: 11 *D. dussumieri*, five *D. arnoldi*, eight *D. hololissa*, and eight individuals not identified to species (see Table 1 for sources of samples). For the cyt *b*, eight *D. dussumieri* were sequenced, two *D. arnoldi*, five *D. hololissa*, and eight unidentified *Dipsochelys* individuals. For the 12S and 16S, three individuals from each *Dipsochelys* species were sequenced. Because we found no variation among the 15 individuals sequenced for the cyt *b* gene, which evolves more rapidly than the ribosomal genes, we sequenced just three individuals per species for both the 12S and 16S. Two *G. elegans* individuals and one *G. sulcata* were sequenced for all mtDNA genes examined. Sequences for *P. arachnoides*, *P. planicauda*, *G. yniphora*, *G. radiata*, *G. pardalis*, *G. nigra* and *G. polyphemus* were obtained from GenBank (Accession Nos. AF020879-AF020899).

2.2. Sequencing methods

DNA was extracted from blood samples using the Easy DNA Kit (Invitrogen). DNA was extracted from

Table 1
Origins of *Dipsochelys* samples

ISIS	Local ID	Species[a]	Origin
	Stan	*D. arnoldi*	NPTS Silhouette
	Clio	*D. arnoldi*	NPTS Silhouette
	Hector	*D. arnoldi*	NPTS Silhouette
T1075	CR82	*D. arnoldi*	Sedgwick Zoo, USA
T1107	6137	*D. arnoldi*	Sedgwick Zoo, USA
	C	*D. dussumieri*	IDC Silhouette
	G	*D. dussumieri*	IDC Silhouette
	T	*D. dussumieri*	IDC Silhouette
T1395	900194	*D. dussumieri*	Honolulu Zoo, USA
T1396	900195	*D. dussumieri*	Honolulu Zoo, USA
T1397	900196	*D. dussumieri*	Honolulu Zoo, USA
T1398	900197	*D. dussumieri*	Honolulu Zoo, USA
T1171	318	*D. dussumieri*	Phoenix Zoo, USA
T1050	7620	*D. dussumieri*	Phoenix Zoo, USA
T1331	11487	*D. dussumieri*	Tulsa Zoo, USA
T1368	11006	*D. dussumieri*	Tulsa Zoo, USA
	Eve	*D. hololissa*	NPTS Silhouette
	Christopher	*D. hololissa*	NPTS Silhouette
	Phoenix	*D. hololissa*	NPTS Silhouette
T1038	2277	*D. hololissa*	Detroit Zoo, USA
T1039	2278	*D. hololissa*	Detroit Zoo, USA
T1392	900191	*D. hololissa*	Honolulu Zoo, USA
T1394	900193	*D. hololissa*	Honolulu Zoo, USA
T1107	6138	*D. hololissa*	Sedgwick Zoo, USA
T1028	300008	?	Loiusville Zoo, USA
T1383	300447	?	Loiusville Zoo, USA
	300494	?	Loiusville Zoo, USA
T1158	300419	?	Loiusville Zoo, USA
T1384	300446	?	Loiusville Zoo, USA
T1404	300493	?	Loiusville Zoo, USA
T1305	3308	?	Phoenix Zoo, USA
T1149	10506	?	Tulsa Zoo, USA

[a] Species assignments made by J. Gerlach based on morphology.

tissue samples of *G. elegans* using the DNEasy Tissue Kit (Qiagen). Standard manufacturer's protocols were used. The primer pair L1091 + H1478 (Kocher et al., 1989) was used to amplify 403 bp of the 12S rRNA gene. The primer pair 16Sar + 16Sbr (Palumbi et al., 1991) was used to amplify 568 bp of the 16S rRNA gene. Primer pair *cyt b* GLU (Pääbo, 1990) + *cyt b* B2 (Kocher et al., 1989) was used to amplify 386 bp of the cyt *b* gene.

Double-stranded PCRs were performed on a Touchdown thermal cycler (Hybaid). PCR conditions were as given in Caccone et al. (1999). Primer dimers and unused nucleotides were removed by cutting DNA bands from a 1% agarose gel and PCR products were purified using a Geneclean Kit (Bio 101, Carlsbad, CA). Automatic sequencing was done on an ABI Prism 377 DNA Sequencer using Big Dye terminators (Perkin–Elmer Cetus) following manufacturer's protocols. Sequencing reactions were run on a 9700 Thermocycler (Perkin–Elmer Cetus). For each individual, strands were sequenced in both directions. Sequences were deposited in GenBank (Accession Nos. AY081779–AY081793).

2.3. Sequence analysis

Sequences were cleaned using Sequencher 4.1 (Gene Codes, Ann Arbor, MI), aligned by using CLUSTAL W (Thompson et al., 1994), and checked by eye. Aligned sequences were analyzed using maximum parsimony (MP; Farris, 1970), maximum likelihood (ML; Felsenstein, 1981), neighbor-joining (NJ; Saitou and Nei, 1987), and Bayesian methods (Huelsenback et al., 2000; Larget and Simon, 1999; Mau and Newton, 1997; Mau et al., 1999; Ranala and Yang, 1996). The three genes were analyzed both separately and combined. *G. polyphemus* was designated as the outgroup. Phylogenetic reconstruction using MP, ML, and NJ was performed using PAUP* 4.0b8 (Swofford, 2000). Bayesian phylogenetic analysis was carried out using MrBayes (Huelsenbeck, 2000).

The number of pairwise substitutions between all combinations of taxa was plotted against Tamura and Nei (1993) distances to produce saturation curves. Transitions (Ti) and transversions (Tv) were examined separately for the 12S, 16S, and each codon position for cyt *b*. Cyt *b* third codon positions were also plotted against Kimura (1980) two-parameter distances because several of the Tamura and Nei distances for this data set were undefined.

For MP analysis, the branch-and-bound search (Hendy and Penny, 1982) was used with ACCTRAN (accelerated transformation) character-state optimization (Swofford, 2000). Various weighting methods were used, including all substitutions unweighted, transversions weighted three times transitions (Tv3×Ti), and transversions only (Tv). For cyt *b*, transitions were also excluded from third codon positions.

For ML analysis, two models of sequence evolution were employed. The HKY85 model (Hasegawa et al., 1985; Yang, 1994) allows different rates for Ti and Tv and different stationary frequencies for each nucleotide. The proportion of invariable sites, the gamma shape parameter, and the Ti/Tv ratio were determined empirically for each gene and for the combined data set. The GTR model (Lanave et al., 1984; Rodríguez et al., 1990; Tavaré, 1986) was employed on the combined data set. This model allows for different rates of change between each nucleotide pair. Rate variation was set as site-specific with partitions designated by gene and, for cyt *b*, by codon position. For all ML analyses, sequences were added randomly with 100 replicates and the TBR branch-swapping algorithm was used.

For the NJ analysis, distances were calculated using the Jukes and Cantor (1969) method, the Kimura (1980) two-parameter method, and the Tamura and Nei (1993) method using an empirically determined gamma parameter. The Jukes and Cantor method was used to obtain distances based on all positions and for Tv only. The Kimura two-parameter method and the Tamura

and Nei method were used to obtain distances for all positions. PAUP* was used to build NJ trees from the distance matrices.

Bayesian analysis, which relies upon the calculation of posterior probabilities for trees, given the data and the specified model of evolution, was carried out using the GTR model with site-specific rate variation partitioned by gene and, for cyt *b*, by codon position. MrBayes was used to run 2,000,000 generations, with a sampling frequency of 100 generations. The MrBayes program relies upon the Metropolis-coupled Markov Chain Monte Carlo method to search tree space for optimal trees. From the 20,000 trees found, the first 1000 were discarded to ensure that a stable likelihood had been reached. The remaining 19,000 trees were used to construct a 50% majority-rule consensus tree using PAUP*.

The bootstrap method (Felsenstein, 1985) was used to test the robustness of the phylogenetic hypotheses generated by MP, ML, and NJ analyses. MP and NJ trees were tested with 1000 bootstrap pseudo-replicates and ML trees were tested with 300 pseudo-replicates. The reliability of each node in the Bayesian tree was determined by calculating the percentage of trees found by MrBayes that contained that grouping. The overall reliability of the Bayesian tree was assessed by determining the percentage of trees that had the same topology as the consensus tree. For the unweighted MP tree for the combined data set, decay index (Bremer, 1988) values were calculated.

Competing phylogenies were compared using the Templeton (1983) test, two-tailed Wilcoxon's rank-sum test (Larson, 1994) and the Shimodaira–Hasegawa log-likelihood test (Shimodaira and Hasegawa, 1999). MP and ML trees from the combined data set were compared with trees derived from the other methods of reconstruction and with constraint trees constructed to test alternate phylogenetic hypotheses regarding the origin of *Dipsochelys*. The resampling estimated log-likelihood (RELL) technique was used in the implementation of the Shimodaira–Hasegawa tests. Templeton tests and Shimodaira–Hasegawa tests were carried out in PAUP*.

2.4. Relative rates tests

Two methods were employed to test whether the rate of sequence evolution among the ten taxa examined has been uniform (i.e., the molecular clock hypothesis). We used the Tajima (1993) test to check for rate homogeneity using the 1D method (Ti + Tv; Ti and Tv separately) and the 2D (Ti + Tv) methods. The three genes were tested separately as were the three codon positions for cyt *b*. All combinations of two Malagasy species were tested with each non-Malagasy species, *G. elegans*, *G. pardalis*, and *G. sulcata*, with non-Malagasy species designated as outgroups. A total of 720 tests were carried out and rate homogeneity was rejected for $P < 0.05$. Tajima tests were carried out using the program MEA, written by Etsuko Moriyama (University of Nebraska).

We also used the likelihood ratio test (Goldman, 1993) to check rate homogeneity for the combined data. The log-likelihood for the ML (HKY85) tree without assuming a molecular clock and the log-likelihood for the same tree assuming a molecular clock were obtained using PAUP*. The likelihood ratio statistic was calculated as $2^*(\ln L_{\text{no clock}} - \ln L_{\text{clock}})$, which follows a chi-square distribution with $n - 1$ degrees of freedom, where n is the number of taxa (Xia, 2000). The null hypothesis that all lineages are evolving at the same rate for the combined data was rejected for $P < 0.05$. This method was employed for all species examined and for all species minus the two *Pyxis* species, which appeared suspicious based on the results of the Tajima rate tests. Approximate divergence dates were estimated based on a rate of sequence divergence of 0.4–0.6% per million years for Testudines mtDNA (Caccone et al., 1999).

3. Results

3.1. Sequence variation

Our analysis was conducted on 403 bp from the 12S, 553 bp from the 16S, and 386 bp from the cyt *b* of each tortoise species. This corresponds to 1,342 bp for each species and a total of 16,104 bp of DNA (both strands were sequenced). All *Dipsochelys* sequences were identical for all individuals for each of the three genes sequenced. Table 2 shows a summary of the sequence data for all taxa, including the percent variable and informative sites, percentage A + T composition, and range of Ti/Tv ratios for each gene and for the combined data set. As expected, the most variable subset of the data was the cyt *b* third codon positions with 63% of sites variable and the least variable was the cyt *b* second codon positions with 6% of the sites variable. Overall, 30% of sites for the cyt *b* was variable, 22% of sites for the 12S, and 20% of sites for the 16S. The percentage A + T remains fairly constant across all genes. The total number of pairwise substitutions, transversions, and Tamura and Nei distances for the combined data is shown in Table 3. Tamura and Nei distances range from 0.07 between *P. arachnoides* and *P. planicauda* to 0.31 between *Gopherus* and *P. arachnoides*.

Saturation curves show that the 12S gene has not reached saturation, the 16S is close to saturation only for the comparisons with the outgroup, the cyt *b* first and second codon positions are not saturated, and the cyt *b* third codon positions have reached saturation (data available from the authors). Traditional wisdom holds that in situations where saturation is evident,

Table 2
Percent variable and informative sites by gene and codon position, A + T percentages, and ranges of transition to transversion ratios for all pairwise combinations of the ten tortoise species examined

Region	Number of sites	Percent variable	Percent informative	% A + T	Ti/Tv
12S	403	22	10	58	17.0–1.86
16S	553	20	12	57	8.75–1.33
cyt *b*	386	30	16	59	29.0–1.93
1st positions	129	19	8	59	8.0–no Tv
2nd positions	129	7	2	60	3.0–no Tv
3rd positions	128	63	38	57	23.0–no Tv
Combined	1342	23	12	58	6.73–1.96

Table 3
Pairwise distances for combined data set

		1	2	3	4	5	6	7	8	9	10
1	*Dipsochelys*	–	97/23	68/13	88/20	102/23	90/15	91/19	96/22	96/16	139/41
2	*G. sulcata*	0.13	–	99/20	105/28	83/13	87/18	118/29	116/32	104/26	134/42
3	*G. radiata*	0.08	0.14	–	75/13	95/22	90/11	88/20	86/23	85/12	136/43
4	*G. yniphora*	0.11	0.15	0.10	–	107/28	104/21	108/28	113/29	95/20	136/46
5	*G. elegans*	0.14	0.11	0.13	0.16	–	85/19	121/32	118/35	80/25	132/46
6	*G. nigra*	0.12	0.12	0.12	0.15	0.11	–	119/21	111/24	95/14	128/41
7	*P. arachnoides*	0.12	0.19	0.11	0.16	0.20	0.19	–	60/12	120/21	158/48
8	*P. planicauda*	0.14	0.18	0.12	0.19	0.19	0.18	0.07	–	113/23	153/49
9	*G. pardalis*	0.13	0.15	0.11	0.13	0.09	0.14	0.20	0.18	–	131/38
10	*Gopherus*	0.24	0.22	0.23	0.22	0.22	0.20	0.31	0.29	0.22	–

Note. Above diagonal—total number of substitutions followed by the number of transversions. Below diagonal—Tamura and Nei distances.

rapidly evolving sites, such as transitions in mtDNA, should be downweighted or omitted for phylogenetic analysis. However, some studies have found that despite appearing saturated, these sites can be phylogenetically informative (see Baker et al., 2001; Yang, 1998; Yoder et al., 1996; Yoder and Yang, 2000). Therefore, both weighted (Tv3×Ti) and unweighted MP analyses were carried out. In addition, transitions were excluded from third codon positions for cyt *b*.

3.2. Phylogenetic analysis

Because all *Dipsochelys* sequences were identical, we included only the data from *D. dussumieri* in the analysis to decrease the computation time. For all methods of phylogenetic analysis employed, *Dipsochelys* was embedded within the Malagasy clade, which includes *P. arachnoides*, *P. planicauda*, *G. radiata*, and *G. yniphora*. For the combined data, two basic topologies for the Malagasy–*Dipsochelys* clade were recovered. The Bayesian tree, the ML trees from the HKY85 model (estimated gamma shape = 0.519803, estimated $Ti/Tv = 4.458203$, estimated proportion of invariable sites = 0.463748) and the GTR model, and the weighted MP bootstrapped tree placed *Dipsochelys* as sister taxon to the *Pyxis* species and grouped *G. radiata* with *G. yniphora* (Figs. 2A and B). The ML trees and the weighted MP tree were identical in topology while the Bayesian tree differed in its placement of *G. pardalis*. The unweighted MP tree and the NJ tree based on Tamura and Nei distances resulted in a different grouping, placing *G. radiata* as sister taxa to the *Pyxis* species, *Dipsochelys* as sister taxa to the *Pyxis*–*G. radiata* clade, and *G. yniphora* as basal to the Malagasy radiation (Figs. 3A and B). All methods of analysis for all genes and for the combined data strongly supported the grouping of the *Pyxis* species and all methods supported the monophyly of the Malagasy tortoises plus *Dipsochelys*.

Templeton tests and Shimodaira–Hasegawa tests were carried out to determine if the topologies recovered under the different methods of analysis are significantly different from one another and to test competing hypotheses regarding the origin of *Dipsochelys*. As Goldman et al. (2000) point out, the Templeton test and the Kishino–Hasegawa test (Kishino and Hasegawa, 1989) are intended for use with trees derived independently from the data and specified a priori. For this reason, we have used the Shimodaira–Hasegawa test for comparing likelihood topologies instead of the Kishino–Hasegawa test. The Shimodaira–Hasegawa test is a nonparametric test similar to the Kishino–Hasegawa test that correctly compares different trees derived from analysis of a single data set (Goldman et al., 2000). To our knowledge, the performance of the Templeton test under such circumstances has not been thoroughly evaluated and a more suitable alternative for comparing parsimony topologies has not been developed. Therefore, although the Templeton test is generally considered to be a conservative

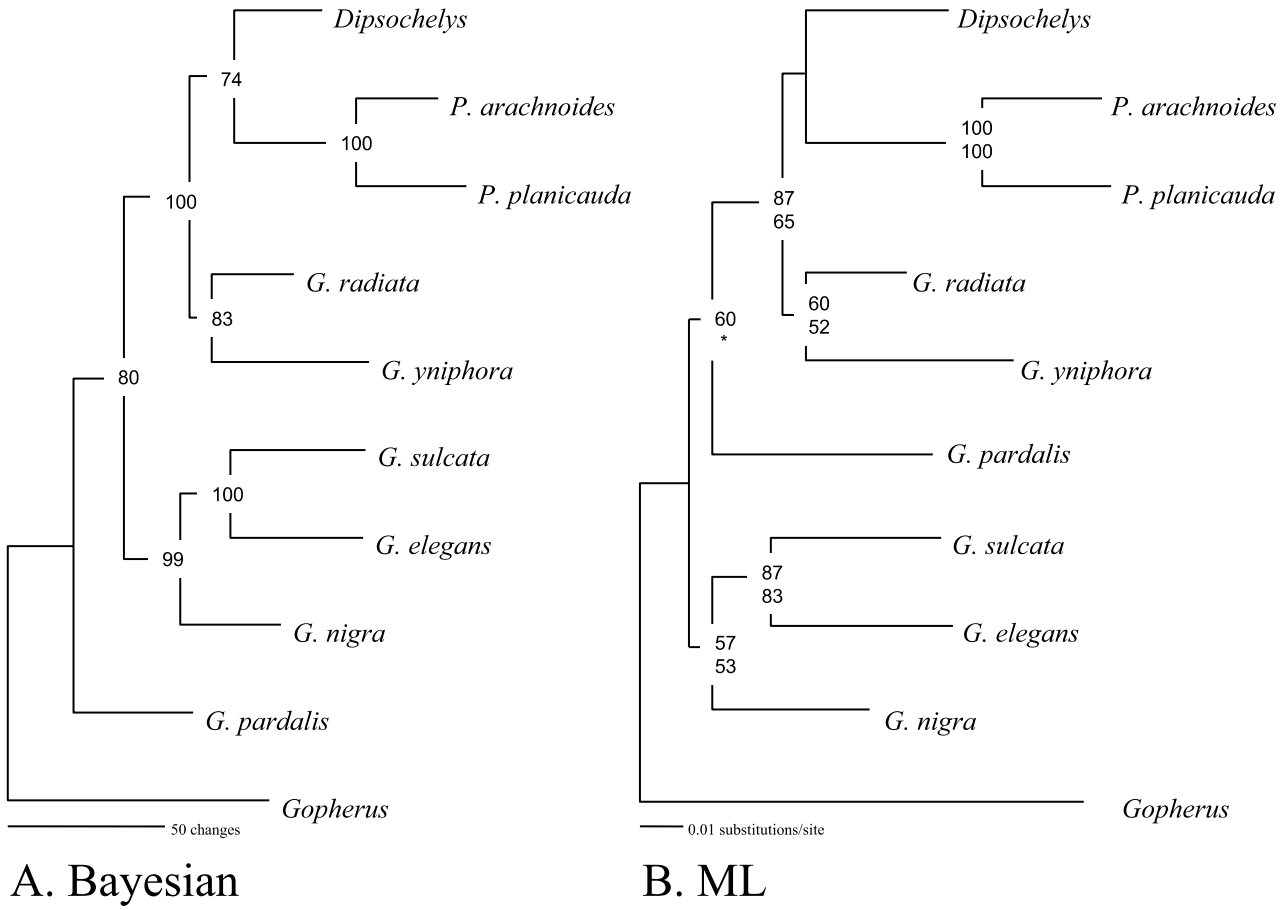

Fig. 2. (A) Bayesian tree generated from a 50% majority rule consensus of 19,000 trees. Numbers on nodes represent the percentage of trees that contained that grouping. Overall, 61% of trees found had the same topology as the consensus tree. (B) ML (HKY85) tree. The topologies of the ML (GTR) tree and the weighted (Tv3×Ti) MP bootstrap tree were identical to this topology. Numbers represent bootstrap values for ML (top) and weighted MP (bottom). Only bootstrap values above 50% are reported. * Bootstrap value less than 50% for weighted MP.

test, its results in this analysis should be interpreted with some caution.

Table 4 shows that the trees derived from the different methods of analysis are not significantly different. Constraint trees were constructed to test alternate topologies. In each case, *Dipsochelys* was constrained to be a sister taxon of *G. sulcata* (C1), *G. elegans* (C2), *G. pardalis* (C3), and *G. nigra* (C4), while leaving the topology of the rest of the tree unchanged. When testing parsimony trees using the Templeton test, all of the constraint topologies were significantly different from the best tree, supporting the hypothesis that *Dipsochelys* is contained within the Malagasy grouping. When testing likelihood trees using the Shimodaira–Hasegawa test, the constraint trees grouping *Dipsochelys* with *G. pardalis* (C3) and the constraint tree grouping *Dipsochelys* with *G. nigra* (C4) under the HKY85 evolutionary model were not significantly different from the ML tree. All other constraint trees, including those grouping *Dipsochelys* with *G. sulcata* (C1) and *G. elegans* (C2), were significantly different from ML trees.

3.3. Relative rates tests

To test the assumption of rate constancy among lineages, we employed Tajima (1993) relative rates tests, using the 1D and the 2D methods. A summary of the results is given in Table 5. Genes were tested separately as were the codon positions for the cyt *b*. A total of 720 tests were conducted. Overall, 7% of Tajima tests was significant, indicating a violation of the molecular clock hypothesis. For the 12S, 120 tests were conducted with about 17% being significant; for the 16S, 120 tests were conducted with 2.5% being significant; and for the cyt *b*, 480 tests were conducted with about 6% being significant. The significant tests were not distributed randomly throughout the data set, however. The *Pyxis* species were involved in all but two of the significant tests. The two significant tests that did not involve either *P. arachnoides* or *P. planicauda* involved *Dipsochelys*, *G. radiata*, and *G. elegans* for the 16S 1D (Ti + Tv and Tv) tests. Interestingly, no tests involving both *Pyxis* species with any outgroup were significant. This suggests that, although the rate of sequence evolution in *Pyxis* is not

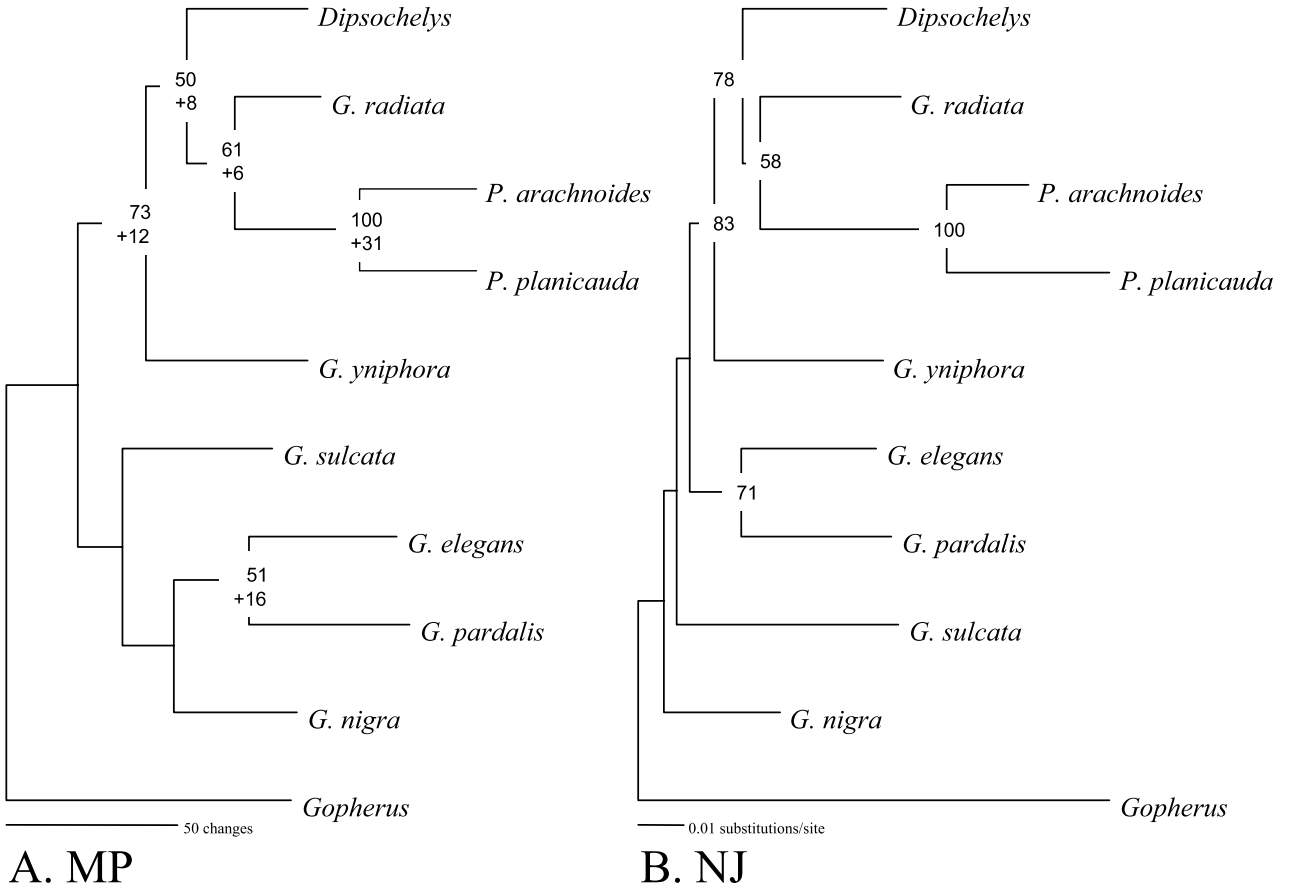

Fig. 3. (A) Unweighted MP tree. Numbers represent bootstrap values (top) and decay indices (bottom). (B) NJ tree constructed from Tamura and Nei distances. Only bootstrap values over 50% are reported.

Table 4
P values for Templeton tests and Shimodaira–Hasegawa tests for various tree topologies, including MP, ML, NJ, and Bayesian trees. Constraint trees (C1–C4) were constructed to test alternate hypotheses concerning the origin of *Dipsochelys*

Tree	Templeton test		Shimodaira–Hasegawa test	
	Maximum parsimony		Maximum likelihood	
	Unweighted	Weighting Tv3×Ti	HKY85	GTR
MP Unweighted	(Best)	0.429	0.149	0.311
ML/MP Ti3×Tv	0.402	(Best)	(Best)	(Best)
NJ-TN	0.549	0.216	0.134	0.179
Bayesian	0.284	1.000	0.828	0.86
C1: (*Dipsochelys*, *G. sulcata*)	0.007*	0.002*	0.001*	<0.001*
C2: (*Dipsochelys*, *G. elegans*)	<0.001*	0.003*	0.001*	<0.001*
C3: (*Dipsochelys*, *G. pardalis*)	<0.001*	0.008*	0.247	0.173
C4: (*Dipsochelys*, *G. nigra*)	0.003*	0.040*	0.055	0.025*

*Tree topology is significantly different from the best tree at $\alpha = 0.05$.

consistent with the rate of the other species tested, it is constant within *Pyxis*.

The likelihood ratio test was employed to further investigate the rate homogeneity. The likelihood ratio statistic (δ) was calculated using likelihoods from the HKY85 model. With all taxa included, $\delta = 55.04$. This value is much greater than the chi-square critical value ($\alpha = 0.05$) of 16.919 for df = 9. Thus, the molecular clock hypothesis must clearly be rejected for all taxa. Because the results of the Tajima tests indicated that the *Pyxis* species may be to blame, the likelihood ratio test was conducted excluding them. In this case, $\delta = 8.59$, which is less than the chi-square critical value ($\alpha = 0.05$) of 14.067 for df = 7. Therefore, when *Pyxis* is excluded, rate homogeneity among lineages is maintained.

Table 5
Percentage of Tajima rate tests that were significant ($\alpha = 0.05$), indicating a non-uniform rate of sequence evolution, and the percentage of significant tests involving either of the *Pyxis* speices

		12S	16S	Cyt *b*			
				All Positions	1st Positions	2nd Positions	3rd Positions
1D	Ti + Tv	27	3	13	0	0	17
	Ti	7	7	10	0	0	10
	Tv	17	0	13	0	0	3
2D	Ti + Tv	17	0	13	0	0	10
Percentage involving *Pyxis*		100	33	100	0	0	100

Note. No tests involving both *Pyxis* species were significant.

To find approximate dates for the appearance of *Dipsochelys* and the colonization of Madagascar by tortoises, the combined absolute distance data were used to estimate divergence times based on a rate of sequence divergence of 0.4–0.6% per million years (Caccone et al., 1999). To avoid violating the molecular clock assumption, the *Pyxis* species were eliminated from the analysis. The tree used for divergence time estimation was recovered from an ML (HKY85) search using the combined data excluding *Pyxis*. The topology of this tree was identical to the ML tree shown in Fig. 2 B minus *Pyxis*. Our estimates suggest that the divergence time of the Malagasy clade, and thus the colonization of Madagascar by tortoises, occurred about 17.5–11.5 myr ago and the split between the ancestor of *Dipsochelys* and the ancestor of *G. radiata–G. yniphora* occurred about 14.5–9.5 myr ago. This date for the initial colonization of Madagascar by tortoises is slightly younger than the previous estimate of 22–14 myr ago by Caccone et al. (1999).

4. Discussion

Our phylogenetic analysis suggests that *Dipsochelys* shares a common ancestor with modern Malagasy tortoise species. This grouping of *Dipsochelys* with the Malagasy species has also been suggested on the basis of cranial osteology (Gerlach, 2001). Therefore, the colonization of Aldabra, the Seychelles, and possibly the Comoros likely occurred from Madagascar or in a stepwise fashion beginning in Madagascar, as suggested by Arnold (1979). Our tests of alternate phylogenies suggest that it is extremely unlikely that *Dipsochelys* colonized the western Indian Ocean from either Africa or India, the only other plausible sources.

Changing sea levels have had a profound effect on the patterns of colonization of western Indian Ocean islands. Madagascar, the granitic Seychelles, and the volcanic Comoros are high islands which would have remained exposed during rising sea levels associated with late Pleistocene interglacial periods, while Aldabra and its surrounding islands are low coralline islands which would have been completely submerged (Peake, 1971). Fossil data coupled with an analysis of changing sea levels show that giant tortoises colonized the island of Aldabra on at least three separate occasions (Taylor et al., 1979), and that on each occasion, the island was colonized by tortoises indistinguishable by fossils from those alive on Aldabra today (Arnold, 1979). The most recent inundation of Aldabra was 125,000 years ago and the colonization of the present fauna likely occurred 80 to 100,000 years ago (Arnold, 1979). It has been suggested that the present assemblage of reptiles on Aldabra more closely resembles that of Madagascar and the Comoros than that of East Africa or the Seychelles (Arnold, 1976). Combined with our phylogenetic results, the presence of two Malagasy giant tortoises (*D. abrupta* and *D. grandidieri*) known from recent fossils, the repeated colonization of nearby Aldabra, and oceanic currents flowing northward towards Aldabra from the northeast coast of Madagascar (Fig. 1) all provide evidence that *Dipsochelys* giant tortoises did colonize Aldabra from Madagascar.

Favorable ocean currents would also make possible the colonization of the Seychelles from Madagascar or from Aldabra. Unlike Aldabra and its surrounding low islands, the granitic Seychelles have never been completely inundated. Therefore, they need have been only colonized a single time. The majority of the present lizard fauna of the Seychelles are comprised of species with putative origins in Madagascar and the nearby Comoros (Cheke, 1984), demonstrating that over-sea dispersal by reptiles from these areas occurs with some frequency. In addition, it has been suggested that gigantism is a preadaptive trait for long distance colonization of oceanic islands (Pritchard, 1996). Therefore, it seems likely that giant tortoises could have survived the journey from Madagascar or Aldabra to the Seychelles.

Until recently, all tortoises from Aldabra, the Seychelles, and the surrounding islands were referable to a single species, *Geochelone gigantea*. Based on morphological differences, Gerlach and Canning (1998) divided *G. gigantea* into four distinct species, three extant and one extinct, and placed them in the genus *Dipsochelys*. We examined the three extant *Dipsochelys* species and

found no differences among them for the regions of the mtDNA examined, calling into question their true genetic distinctiveness. Analysis of faster evolving regions (control region of mtDNA and microsatellites) is in progress to specifically address the degree of differentiation among these lineages (submitted).

This study and a host of morphological (Bour, 1980; Crumly, 1982; Ernst and Barbour, 1989; Gaffney and Meylan, 1988; Gerlach, 2001; Meylan and Sterrer, 2000) and molecular (Caccone et al., 1999) studies indicate that *Geochelone* is a paraphyletic genus. One potential solution would be to include *Dipsochelys* and *Pyxis* in *Geochelone* thereby creating a large and highly morphologically and ecologically variable genus. Such a solution may be inappropriate, however, because it would obscure the morphological and molecular distinctiveness of *Pyxis* and *Dipsochelys*. Our data indicate that the Malagasy taxa plus *Dipsochelys* could be correctly assigned to a monophyletic taxon, but our sampling of *Geochelone* is inadequate to provide phylogenetic insights at the genus level. A more extensive molecular phylogenetic study including all *Geochelone* species and multiple outgroups is warranted and might be combined with a reanalysis of morphological characters, such as that conducted by Gerlach (2001), to help resolve the taxonomy of this presently confused genus.

Dating speciation events for Malagasy tortoises is challenging due to the lack of fossil material. Madagascar has a spotty fossil record for terrestrial vertebrates, with records from only the late Cretaceous (83–74 myr ago) and the late Pleistocene and Holocene (Krause, 1995). The large intervening temporal gap in the fossil record has made it difficult to reconstruct the biogeography of many vertebrates, including tortoises. The oldest tortoise fossils on Madagascar are just 2850 years old (Mahé and Sourdat, 1973). Therefore, fossil dates are not available to calibrate the molecular clock for dating deeper divergences. Nonetheless, our estimates indicate that Madagascar was colonized by tortoises once, 17.5–11.5 myr ago and that the ancestor of *Dipsochelys* appeared about 14.5–9.5 myr ago.

The earliest fossils of Testudinidae date back to the Eocene, 55–35 myr ago (Auffenberg, 1974). The Madagascar/India landmass separated from Africa about 165 myr ago (Rabinowitz et al., 1983) and Madagascar had reached its current position by 123 myr ago (Agrawal et al., 1992). Therefore, it is unlikely that tortoises existed on Madagascar when it was still attached to Africa. Instead, colonization likely occurred over the Mozambique Channel once Madagascar had reached its current position (Caccone et al., 1999).

India broke from Madagascar about 88 myr ago (Storey et al., 1995) and moved northward until it collided with Asia 45 myr ago (Braithwaite, 1984). The Seychelles, once part of India, remained behind as oceanic islands. Since our molecular analysis suggests that *Dipsochelys* does not share common ancestry with the Indian *G. elegans*, it is unlikely that a *Dipsochelys* ancestor was present on the Seychelles when they became isolated. Instead, colonization of the Seychelles likely occurred over-water, either directly from Madagascar or from a nearby island.

Our results involving the extant tortoises of Aldabra, the Seychelles, Madagascar, and the surrounding continents provide an origin for the radiation of tortoises throughout the western Indian Ocean and point to probable colonization routes. However, a molecular study also including the extinct tortoises of Madagascar, the Comoros, and the Mascarenes would be extremely valuable for reconstructing a more precise pattern of colonization of Indian Ocean islands by giant tortoises (see Austin and Arnold, 2001).

An additional finding of interest is that *Pyxis* appears to have an accelerated rate of mtDNA sequence evolution compared to the other tortoise species examined. The data presented here and several morphological studies indicate that the two *Pyxis* species are closely related and highly derived compared to other Malagasy tortoises (Bour, 1981; Obst, 1978, 1980). Morphologically these species possess several paedomorphisms, or juvenile-like characters, including a partially fused quadrate (Gerlach, 2001) and, for *P. planicauda*, a hinged plastron which may be indicative of delayed ossification (Bour, 1981; Obst, 1978, 1980). The *Pyxis* species are also notably similar in ecology and size. Both inhabit tropical deciduous forests and dry woodlands and neither exceeds a carapace length of 15 cm, making them some of the smallest tortoise species in the world (Ernst and Barbour, 1989). *Pyxis* is also characterized by early sexual maturity and relatively short generation times. *P. arachnoides* reaches maturity by seven years for males and by eleven years for females (Jesu and Schimmenti, 1995). In comparison, *D. dussumieri* does not reach sexual maturity in the wild until twenty to twenty-five years of age (J. Gerlach, unpublished data).

Metabolic rate, body size, and generation time have all been correlated with mtDNA rate variation in vertebrates. However, interactions among these factors are still not well understood (see Rand, 1994). The tortoises examined here show a range of body sizes and generation times, while all possessing similar metabolic rates. Therefore, our finding that *Pyxis*, the smallest species examined with the shortest generation times, has an increased mtDNA rate indicates that rate variation in vertebrates may be influenced by these factors (or some unidentified correlate) apart from the effects of metabolism.

It is interesting to consider the relatively recent shared ancestry of the smallest and second largest living tortoise lineages in the world. The extremely different morphologies seen in *Pyxis* and *Dipsochelys* must have

arisen since the colonization of Madagascar just 17.5 myr ago and may have arisen as recently as about 10 myr ago (see Section 3). This is an extraordinarily rapid morphological change for turtles, which are generally quite conservative in morphological evolution (see Bickham, 1984). Since tortoise fossils from this period have not been found, the body size of the colonizing ancestor is not known. But based on the superior colonizing ability of the large-bodied *Dipsochelys*, one might speculate that a large-bodied ancestor made its way to Madagascar and gave rise to the diversity of tortoises seen there and once found on islands throughout the western Indian Ocean.

5. Conclusions

Our phylogenetic analysis based on sequences from three mtDNA genes suggests that Indian Ocean giant tortoises (*Dipsochelys*) evolved on Madagascar from a common Malagasy ancestor and colonized the islands of the western Indian Ocean from Madagascar and its nearby islands. Aldabra and its surrounding coral islands were repeatedly colonized following inundation in the late Pleistocene and ocean currents flow directly towards Aldabra from the northeast coast of Madagascar. Therefore, it is likely that giant tortoises colonized Aldabra from Madagascar and it is probable that the Seychelles were colonized from Madagascar, Aldabra, or a surrounding island. It has previously been suggested that Aldabra was colonized from the Seychelles (Gerlach and Canning, 1998), but based on our data and ocean currents that favor transport to the Seychelles from Aldabra rather than vice versa (Cheke, 1984), this hypothesis seems unlikely. We found no sequence variation among the three extant *Dipsochelys* species examined, calling into question the true distinctiveness of these lineages. Additionally, an accelerated rate of mtDNA evolution was detected in *Pyxis* compared to the other tortoise species examined. This rapid rate of genetic change may be associated with the small body sizes and short generation times that characterize this genus.

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