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Evolution

A molecular phylogeny for marine turtles: Trait mapping, rate assessment, and conservation relevance

(conservation genetics/cytochrome *b*/molecular systematics/mitochondrial DNA)

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ABSTRACT **Nucleotide sequences from the cytochrome *b* gene of mitochondrial DNA were employed to resolve phylogenetic controversies and to assess molecular evolutionary rates in marine turtles (Chelonioidea). Findings of special relevance to conservation biology include discovery of a distant relationship between *Natator* and other cheloniid species, the paraphyly of *Chelonia mydas* with respect to *Chelonia agassizi*, and genetic distinctiveness of *Lepidochelys kempi* from *Lepidochelys olivacea*. A longstanding debate in evolutionary ecology was resolved by phylogenetic mapping of dietary habits, which indicates that the spongivore *Eretmochelys imbricata* evolved from a carnivorous rather than a herbivorous ancestor. Sequence divergences at intergeneric and interfamilial levels, when assessed against fossil-based separation times, support previous suggestions (from microevolutionary comparisons) that mitochondrial DNA in marine turtles evolves much more slowly than under the "conventional" vertebrate clock. This slow pace of nucleotide replacement is consistent with recent hypotheses linking substitution rate to generation length and metabolic pace.**

Turtles (order Testudines) first appear in the fossil record some 200 million years ago (mya), and by 150 mya fully marine forms made their appearance (1). However, the subsequent evolution of marine turtles has been a matter of much speculation and debate, as is reflected in uncertainties about evolutionary relationships at taxonomic levels ranging from subspecies to suborders (Table 1). Previous hypotheses regarding the phylogeny of marine turtles (Fig. 1) have rested primarily on morphologic characters and a reasonably abundant fossil record. Here we provide an independent assessment of evolutionary relationships among all eight extant species, based on nucleotide sequences from the cytochrome *b* gene of mitochondrial DNA (mtDNA).

One motivation for this study is to clarify marine turtle phylogeny in problematic areas that are relevant to the fields of both evolutionary ecology and conservation genetics (Table 1). For example, the molecular phylogeny is used to decipher the evolutionary origin of an unusual dietary habit of the hawksbill turtle, spongivory. Furthermore, all of the marine turtle species are formally listed by the International Union for the Conservation of Nature and Natural Resources as threatened or endangered, and by enhancing phylogenetic understanding, genetic information may influence strategies for allocating finite management resources. At present, several national and international conservation programs are directed toward various marine turtles whose relationships and even specific status are in question.

A second rationale for this study is to evaluate recent suggestions of a significant slowdown in the evolutionary rate of turtle mtDNA relative to many other vertebrates (6–8).

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Table 1. A recent taxonomy for marine turtles, with problematic areas indicated by parenthetical questions

Order Testudines—all extant turtles, freshwater, terrestrial, and marine
Family Dermochelyidae—*Dermochelys coriacea* (leatherback)
(Are marine turtles monophyletic? Where does this species lie with regard to broader turtle phylogeny?)
Family Cheloniidae
Tribe Chelonini
Genus *Chelonia*—*C. mydas* (green)
C. agassizi (black)
(Are these two forms distinct species?)
Genus *Natator*—*N. depressus* (flatback)
(Is this species a close ally of the green turtle, or perhaps allied more closely to members of the Carettini?)
Tribe Carettini
Genus *Caretta*—*C. caretta* (loggerhead)
Genus *Lepidochelys*—*L. olivacea* (olive ridley)
L. kempi (Kemp's ridley)
(Are these two forms distinct species?)
Genus *Eretmochelys*—*E. imbricata* (hawksbill)
(Is this spongivorous species allied more closely to the carnivorous Carettini or to the herbivorous Chelonini?)

These suggestions were based on restriction-site comparisons at intraspecific and intrageneric levels in turtles, where points of separation were dated by zoogeographic evidence. Here, direct nucleotide sequencing and divergence times from fossil evidence are employed to calibrate molecular rates across much deeper evolutionary nodes.† The confirmation of an unusually slow pace of molecular evolution in marine turtles would support recent suggestions linking nucleotide substitution rates to generation length and metabolic rate (6, 9–11).

MATERIALS AND METHODS

To address these evolutionary issues, mtDNAs from all extant species of marine turtles were isolated by CsCl density gradient centrifugation (12) or phenol extraction (for *Chelydra serpentina*). Cytochrome *b* sequences were amplified by the polymerase chain reaction (13, 14), using two primer pairs that produce fragments that overlap by 100 base pairs (bp): CB1-L and CB2-H, which generate fragments of length ≈307 bp, and CB4-L and CB3-H, which amplify fragments ≈500 bp long (ref. 15; see also ref. 16). Fragments were checked for correct size by electrophoresis in 1% agarose gels and then

Abbreviation: mya, million years ago.

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L12712–L12720, L12762–L12764, and L13389).

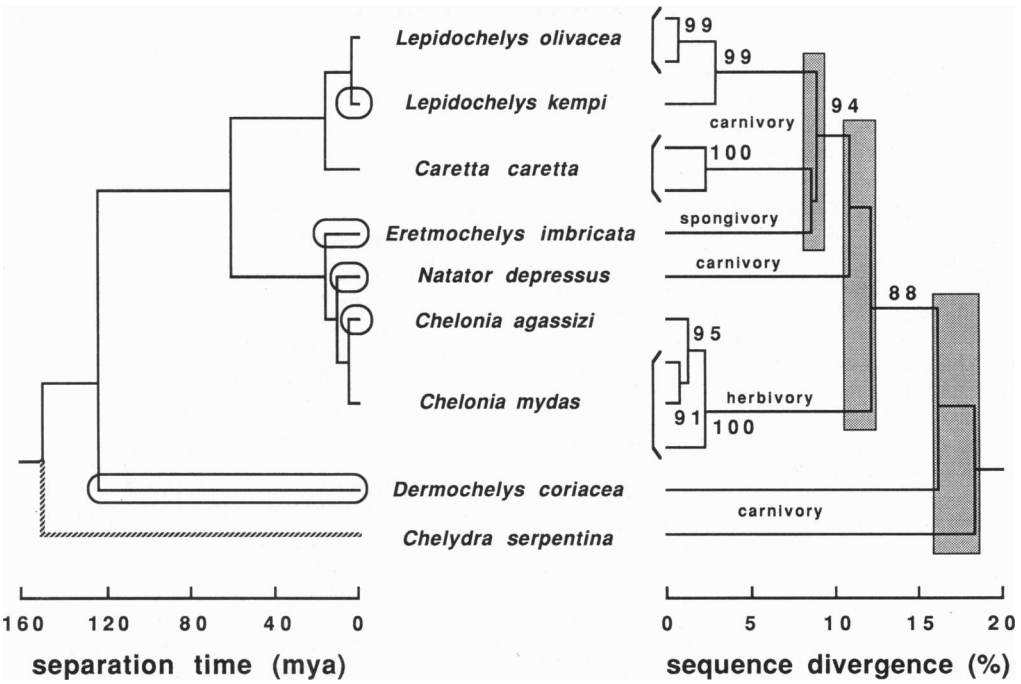

FIG. 1. Evolutionary relationships among marine turtles and a putative outgroup, *Chelydra serpentina*. (*Left*) Phylogenetic assessment based on morphologic characters as presented by ref. 2 (see also ref. 3) and modified to include a species-level distinction of *Chelonia agassizi*. An approximate time scale based on fossil evidence (see text) is shown. Ellipses identify controversial regions in the phylogeny. (*Right*) Phylogenetic summary based on analyses of the mtDNA sequence data. In this composite phylogeny [the basic framework of which is, for simplicity, a UPGMA (unweighted pair-group method with arithmetic means) dendrogram], putative branching orders that received limited support in alternative methods of analysis (e.g., <85% under bootstrapping, or those that varied with data base or phylogenetic algorithm employed) are conservatively depicted as unresolved trichotomies (shaded areas). For example, *Natator* usually falls just outside the Carettini–Chelonini group under various parsimony analyses, whereas under UPGMA and neighbor joining it allies weakly with the Carettini or Chelonini, respectively, and *Dermochelys* groups with the other marine turtles under UPGMA but with *Chelydra* under neighbor joining and some of the parsimony analyses. On the figure, bootstrap values (from parsimony analyses) for the well-supported clades are based on the entire data set for intrageneric comparisons (where sequence divergences were <5%) and on transversions alone for the higher taxonomic levels (where sequence divergences were >5%) (see ref. 4). Distances in the genetic scale shown are based on ref. 5.

separated from excess primers and dNTPs with use of the Magic PCR Preps system from Promega. Direct sequencing of heat-denatured, double-stranded amplification products (15) was carried out by dideoxy chain termination (17), using T7 DNA polymerase (Sequenase, Version 2.0, United States Biochemical) and ^{35}S labeling. To resolve ambiguities and assure accurate sequence information, both the light and heavy strands were sequenced from each individual. Alignment of all nucleotide sequences was unambiguous. Where possible, two or three conspecific individuals representing separate ocean basins were included: *Caretta caretta*, Atlantic and Indian Oceans; *Lepidochelys olivacea*, Atlantic and Pacific Oceans; *Chelonia mydas*, Atlantic, Pacific, and Indian Oceans. The freshwater snapping turtle (*Chelydra serpentina*), postulated to be a close extant relative of the marine turtles (3), also was assayed.

Sequence divergence estimates were calculated as direct counts of nucleotide sequence differences, and also by the "two-parameter" method (5) to correct for multiple substitutions at a site (using an empirically based transition/transversion ratio of 3.0). Evolutionary relationships were estimated by a variety of procedures in the computer programs PAUP (18) and PHYLIP (19). These involved distance matrix methods [UPGMA clustering (20) and neighbor joining (21)], as well as maximum-parsimony methods applied to qualitative data coded in each of two formats: (*i*) nucleotide sequences themselves and (*ii*) purines versus pyrimidines (so that resulting phylogenies reflect transversional changes only). (Data also were coded as translated amino acid sequences, but the relatively small number of replacement substitutions observed inhibited strong resolution of most clades.) For each parsimony analysis, phylogenies were estimated by using the branch-and-bound search option, and the degree of support was evaluated with 200 bootstrapping replicates.

RESULTS AND DISCUSSION

Molecular Phylogeny of Marine Turtles. Cytochrome *b* sequences from 13 specimens are available from GenBank (see Introduction) or from B.W.B. The sequence used for this analysis begins with codon 47 of the cytochrome *b* gene (ref. 16 and references therein). Among the 503 nucleotide positions assayed per individual, 151 were polymorphic across taxa, 59 sites exhibited one or more transversions, and 32 sites involved amino acid substitutions. Sequence divergence estimates (corrected for multiple hits, ref. 5) ranged from 0.006 to 0.136 within Cheloniidae, from 0.146 to 0.178 between Cheloniidae and Dermochelyidae, and from 0.166 to 0.200 in various comparisons with the snapping turtle.

Several widely accepted elements of marine turtle systematics (Table 1) were supported by the molecular phylogenetic analyses (Fig. 1). These include (*i*) a distant position of *Dermochelys* (and *Chelydra*) relative to all other marine turtles; (*ii*) within Cheloniidae, a deep evolutionary separation of the tribe Chelonini (represented by *Chelonia*) and the tribe Carettini (*Caretta* and hypothesized allies); (*iii*) the systematic affiliation of *Lepidochelys* with *Caretta*; (*iv*) the grouping of the two *Lepidochelys* species as sister taxa; and (*v*) the genetic distinction of *L. kempi* from *L. olivacea*. This latter observation agrees with a previous report based on mtDNA restriction sites (8) and is of special conservation relevance because the Kemp's ridley is regarded as one of the world's most endangered vertebrates. The sole remaining

population (in Tamaulipas, Mexico) has been the subject of an intensive international conservation effort, despite questions about the evolutionary distinctiveness of the Kemp's ridley from the globally distributed olive ridley (8).

On the other hand, several discrepancies between the mtDNA phylogeny for marine turtles and "conventional" taxonomy also were apparent:

(*a*) *Chelonia*. The black turtle (*C. agassizi*) inhabits the eastern Pacific Ocean, whereas the green turtle (*C. mydas*) is distributed globally in tropical waters. Some authors recognize *C. agassizi* as a valid species, but others view the black turtle as a poorly defined subspecies or morphotype of the green (see ref. 22). The cytochrome *b* sequences are consistent with previous conclusions from restriction fragment length polymorphism data that *C. mydas* is paraphyletic with respect to *C. agassizi* in terms of matriarchal phylogeny (23). In other words, the eastern Pacific "black turtle" comprises but a small subset of lineage diversity within the broader and deeper mtDNA gene tree for the globally distributed green turtle. Thus the genetic data give added weight to (but cannot prove) Mrosovsky's (22) suggestion that the black turtle may be a melanistic form of the green turtle separated only at the populational level.

(*b*) *Natator depressus*. The flatback turtle, restricted to Australia and adjacent waters, traditionally was considered a close relative of the green turtle and was labeled *Chelonia depressa*. Recently, two independent research groups resurrected the genus *Natator* and suggested that the flatback may be affiliated with Carettini rather than Chelonini (24, 25). A relatively large genetic distance ($P \approx 0.109$) observed between the flatback and green turtles adds support for the resurrection of *Natator* as distinct from *Chelonia*. However, *N. depressus* also exhibits a comparably large mtDNA distance ($P \approx 0.108$) from the Carettini. In the phylogenetic analyses overall (Fig. 1), three major mtDNA lineages are documented within Cheloniidae, but the available molecular data cannot resolve what appears to be a near trichotomy for the Chelonini, Carettini, and *Natator*.

(*c*) *Eretmochelys imbricata*. Spongivory is extremely rare among vertebrates (26). Did the spongivorous hawksbill turtle arise from a carnivorous or herbivorous ancestor? One school of thought maintains that the hawksbill is allied closely to the herbivorous green turtle within Chelonini (2, 3, 25, 27), whereas another school maintains that the hawksbill belongs with the carnivorous loggerhead in Carettini (1, 28–30). All phylogenetic analyses of the mtDNA data support placement of the hawksbill turtle with Carettini rather than Chelonini, thus indicating that the spongivorous feeding habit of *E. imbricata* probably evolved from a carnivorous rather than herbivorous ancestral condition (Fig. 1). Within the Carettini, the exact placement of *Eretmochelys* based on mtDNA is less certain, with various analyses weakly supporting alternative clades and therefore leaving unresolved a near trichotomy for *Eretmochelys, Lepidochelys*, and *Caretta*.

(*d*) *Dermochelys coriacea*. This species is distinguished from other marine turtles by unusual skeletal features, partial endothermy, and a highly modified external morphology (1, 3, 31, 32). Cope (33) erected a suborder (Athecae) to distinguish the shell-less leatherback from all other turtles (marine or otherwise), and this distinction has been championed intermittently throughout the last century (refs. 31 and 32 and references therein). Other researchers maintain that differences between the leatherback and other marine turtles warrant recognition merely at the subfamilial or generic level (reviewed in ref. 1; see also refs. 34 and 35). Phylogenetic analyses of the mtDNA data support a clear distinction of Dermochelyidae from Cheloniidae, but the magnitude of sequence separation relative to that exhibited by the "outgroup" *Chelydra serpentina* appears to contradict Cope's (33) suggestion that the leatherback is the sister taxon to all other living turtles. Phenetic analyses favor a grouping of the extant marine turtles relative to *Chelydra*. However, because the designation of *Chelydridae* as the closest extant family to the marine turtles is somewhat controversial (and because the relevant bootstrapping under parsimony requires multiple outgroups), DNA sequences from many additional species of Testudines and non-turtles will be required to determine whether extant marine turtles are mono- or polyphyletic.

Evolutionary Rates in mtDNA. The mtDNA sequence data also were used to address issues of molecular evolutionary rate. For these purposes, genetic distances were compared against the following provisional evolutionary nodes that previously had been dated from reasonably strong fossil evidence: (*i*) Dermochelyidae versus the proto-Cheloniidae, 100–150 mya (27, 36); (*ii*) Carettini versus Chelonini, 50–75 mya (refs. 36 and 37; but see ref. 27); (*iii*) *Caretta* versus *Lepidochelys*, 12–20 mya (27, 28); and (*iv*) *L. kempi* versus *L. olivacea*, perhaps 4.5–5.0 mya (ref. 38; also see ref. 2).

Fig. 2 plots the observed mtDNA genetic distances against these provisional dates, and compares the results with previously published data on sequences of the mtDNA cytochrome *b* gene in ungulate mammals and dolphins (39). A slower average pace for the evolution of turtle mtDNA is apparent. From the initial slope of the divergence curves, total sequence differences (transitions plus transversions) in the marine turtles appear to accumulate at rates less than

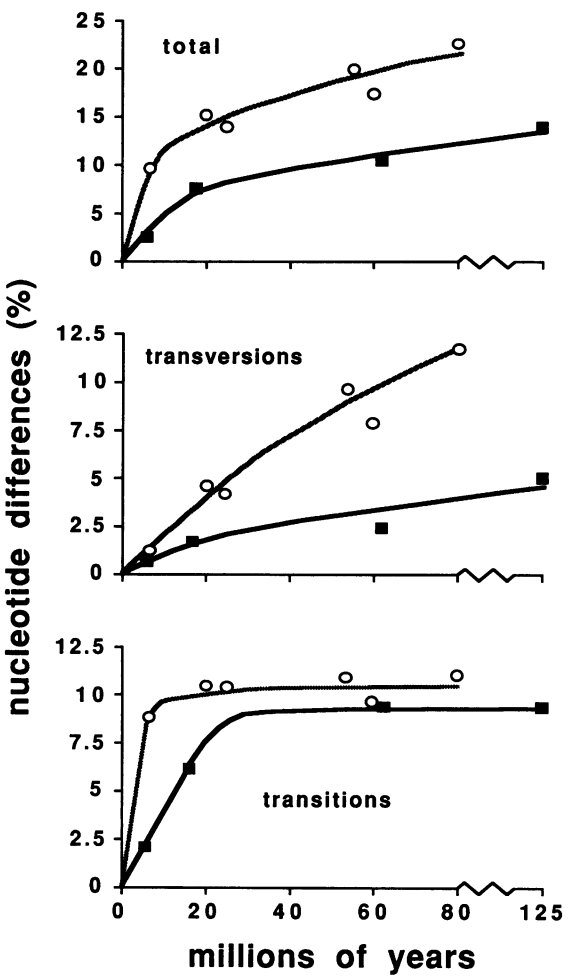

FIG. 2. Dependence of sequence differences (transitions, transversions, and total) between marine turtle taxa (■) and between various ungulate mammals and dolphins (○; after ref. 39). For the turtles, lineage separations were dated from the fossil record (as described in the text). At each divergence time, the point shown is the average of all possible pairwise comparisons.

one-third as great as those in the ungulates (about 0.4% sequence divergence per million years between pairs of lineages in turtles, versus about 1.3% in these mammals). These results support and extend trends previously reported for turtles based on restriction-site comparisons at intraspecific and intrageneric levels (6–8, 23). Furthermore, the lower rates in marine turtles apply to both transitions and transversions (Fig. 2) and to both the cytochrome *b* gene (present study) and the mtDNA molecule overall (as gauged by the earlier restriction-site comparisons). These results suggest that the slow pace of nucleotide substitution in marine turtles is an intrinsic and general feature of their mtDNA, rather than an artifact of differential saturation effects or other confounding factors in the nonlinear process by which mtDNA nucleotide differences accumulate (Fig. 2).

Previous reports have noted a correlation between large body size, slow metabolic rate, long generation time, and slow molecular clocks in several taxonomic groups (6, 9, 10). One proposed mechanism by which such associations might arise invokes the concept of "nucleotide generation time," the average length of time before a nucleotide is copied by replication or repair (9). Metabolic rate and generation time (which also tend to be correlated with body size) may affect substitution rates by altering the mean residence time of a base at a nucleotide position, so that residence times would tend to be shorter in small, short-lived, and metabolically active species. Marine turtles are exceptional examples of long-lived creatures with relatively low metabolic rates, and thus the present molecular results fit well with these rate scenarios. Whatever the reason for the slow molecular rate in turtles, it is increasingly clear that no universal clock for the evolution of vertebrate mtDNA can be assumed in phylogenetic studies.

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