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A SPECIATIONAL HISTORY OF "LIVING FOSSILS": MOLECULAR EVOLUTIONARY PATTERNS IN HORSESHOE CRABS

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Abstract.—Horseshoe crabs' exceptional morphological conservatism over the past 150 My has led to their reputation as "living fossils," but also has served to obscure phylogenetic relationships within the complex. Here we employ nucleotide sequences from two mitochondrial genes to assess molecular evolutionary rates and patterns among all extant horseshoe crab species. The American species *Limulus polyphemus* proved to be the sister taxon to a clade composed of the Asiatic species *Tachypleus gigas, T. tridentatus,* and *Carcinoscorpius rotundicauda,* whose relationships *inter se* were not resolved definitively. Both absolute and relative rate tests suggest a moderate slowdown in sequence evolution in horseshoe crabs. Nonetheless, dates of the lineage separations remain uncertain primarily because of reservations about molecular-clock calibrations resulting from large rate variances at examined loci across Arthropods and other animal lineages, as inferred in this and prior studies. Thus, ironically, separation dates as estimated by molecular evidence in general may remain most insecure in taxonomic groups for which such information is needed most—those lacking strong biogeographic or fossil benchmarks for internal-clock calibrations. In any event, the current results show that large numbers of molecular characters distinguish even these most morphologically conservative of organisms. Furthermore, comparisons against previously published mitochondrial sequence data in the morphologically dynamic hermit crab–king crab complex demonstrates that striking heterogeneity in levels of morphotypic differentiation can characterize Arthropod lineages at similar magnitudes of molecular divergence.

Key words.—Mitochondrial DNA, molecular clocks, phylogeny.

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Evolutionary stasis, the near absence of detectable evolutionary change in some lineages over long periods of geological time, has been perceived as one of the central challenges for modern evolutionary theory (Gould and Eldredge 1977; Williams 1992). In some cases, stasis with regard to morphological appearance also may extend across speciation events, as in the origin of sibling species. In such lineages and clades, what processes confine long-term morphological differentiation within boundaries that are far narrower than they theoretically could be, given the rapid rates of short-term phenotypic change commonly observed in most species under artificial or natural selection? (Williams 1992). Potential explanations range from molecular (e.g., low mutation rate; paucity of additive genetic variation), to ontogenetic (e.g., developmental constraint or coherence producing resistance to selection), to ecological (e.g., long-term stability in environmental selection pressures). Here we examine patterns of molecular evolution in an evolutionary clade renowned for long-term stasis with regard to external morphology.

Because of a reputation for extreme conservatism in morphotypic evolution, horseshoe crabs (Arthropoda; class Merostomata; subclass Xiphosura) have been considered the "archetypes of bradytely [extremely slow evolution]" and "a classic example of arrested evolution" (Fisher 1984). The fossil record indicates that xiphosurans arose and radiated in the early to middle Paleozoic, and that by the mid-Mesozoic some taxa had attained a morphological appearance strikingly similar to that of present-day species (Størmer 1955). Thus, with regard to external morphology, these xiphosuran lineages have remained remarkably unchanged over 150 My or more. As a result, the extant horseshoe crabs often are discussed as paradigm examples of "living fossils" (Eldredge and Stanley 1984) or "phylogenetic relics" (Selander et al. 1970).

At the molecular level, however, living horseshoe crabs appear to be unexceptional with regard to intraspecific genetic variation and patterns of population differentiation. Thus, in *Limulus polyphemus,* the level of allozyme heterozygosity (H = 0.057) proved similar to mean estimates for many other animals (Selander et al. 1970); and, levels and patterns of intraspecific differentiation in mitochondrial DNA (mtDNA) were similar to those of several other inverte-

brate and vertebrate species inhabiting the same coastal range in the southeastern United States (Saunders et al. 1986; Avise 1992). This "normalcy" of genetic variability within and among populations of *L. polyphemus* suggests that consensus patterns of molecular versus morphotypic evolution in horseshoe crabs may be conspicuously decoupled (but see Riska [1981] and Shuster [1982], who documented variability and geographic differentiation in the morphological traits of *L. polyphemus* as well).

Actually, four extant species of horseshoe crabs are recognized: *L. polyphemus* in eastern North America, and *Carcinoscorpius rotundicauda, Tachypleus tridentatus,* and *T. gigas* in Southeast Asia. Despite their placement in three genera, to the untrained eye, the differences in morphology are subtle indeed, consisting of such distinctions as whether the cross-section of the telson (tail) is subtriangular (*C. rotundicauda*) versus triangular (other species), and whether the number of immovable spines on the midposterior margin of the opisthosomatic carapace is three (*T. tridentatus*) versus one (other species). As noted by Selander et al. (1970), generic separation among various living and extinct horseshoe crabs "is a reflection of the morphological differences between them, *relative* to the collection of all forms in the Xiphosura. . . .since taxonomic lines tend to be drawn relative to the range of variation specific to the taxon under consideration." In any event, one consequence of the general morphological conservatism of horseshoe crabs over the past 150 My is that considerable uncertainty exists over the evolutionary histories of the lineages leading to the extant forms. This irresolution of phylogeny as a result of extreme morphological stasis, and questions concerning the magnitude of molecular differentiation in a clade renowned for slow morphological evolution, prompted the current assessment of molecular-level evolutionary patterns among all extant horseshoe crab species.

Materials and Methods

Specimens of *Tachypleus gigas* and *Carcinoscorpius rotundicauda* were collected in the vicinity of Bangsaen (Gulf of Siam), Thailand. Specimens of *T. tridentatus* came from the Bay of Hakata, Fukuoka, Japan. As elaborated below, the two samples of *L. polyphemus* came from genetically distinctive populations along the Atlantic and Gulf of Mexico coastlines in the southeastern United States.

Mitochondrial DNA was isolated from the fresh gill and muscle tissues of *L. polyphemus* by CsCl gradient centrifugation. For the other species, phenolic extractions of genomic DNA were employed. Mitochondrial sequences were amplified via the polymerase chain reaction (Innis et al. 1990; Saiki et al. 1988), using primer pairs 16sar-L and 16sbr-H for the 16S rRNA gene, and CO1f-L and CO1a-H for the cytochrome oxidase I gene (Palumbi et al. 1991). These produced fragments of about 525 bp and 650 bp in length, respectively. Fragments were checked for correct size on 1% agarose gels and then 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 was performed either in our laboratory by dideoxy chain termination using T7 DNA polymerase and ^{35}S radioactive labeling (Sanger et al. 1977), or by fluorescent-dye sequencing conducted by the Molecular Genetics Instrumentation Facility at the University of Georgia. Correctness of DNA sequences was checked by several procedures: first, various portions of the 16S rRNA sequence were assayed both in our laboratory and in the DNA sequencing facility and results compared; second, both heavy and light strands were sequenced from each individual; third, two individuals (A and B) from each species of horseshoe crab were sequenced independently.

The 16S rRNA gene sequences were aligned using the computer program GeneWorks 2.1.1 (Intelligenetics), with assigned penalties of 10 and 4 for opening and extending a gap, respectively. Cytochrome oxidase alignments were unambiguous and done by eye. Estimates of genetic divergence were calculated as direct counts of nucleotide sequence differences, and by the "two-parameter" method of correction for multiple substitutions at a site (Kimura 1980). Distance matrices were clustered by the unweighted pair-group method with arithmetic means (UPGMA; Sneath and Sokal 1973) and by the neighbor-joining procedure (Saitou and Nei 1987). Sequences also were analyzed by maximum parsimony methods as applied to information coded as: (1) nucleotide sequences themselves; (2) purines versus pyrimidines (such that only transversions were considered); and (3) for the cytochrome oxidase locus, first and second positions of codons only (such that silent substitutions at third positions were disregarded). The latter two approaches permit focus on conservative char-

acter-state changes most likely to be phylogenetically informative at deeper evolutionary levels. Both exhaustive and branch-and-bound options were employed in the parsimony searches, and bootstraps were conducted across batches of 100–1000 replicates. The distance-based and parsimony analyses were performed using computer programs PHYLIP (Felsenstein 1991) and PAUP (Swofford 1993), respectively.

For outgroup, we generated and employed an homologous 16S rRNA gene sequence from the scorpion *Vejovis carolinianus* (the cytochrome oxidase primers did not successfully amplify from this species). We also used a GenBank 16S rRNA gene sequence from the brine shrimp *Artemia salina*. The scorpion is an arachnid placed in Chelicerata, the subphylum to which the merostomatid horseshoe crabs belong (Barnes 1963), whereas the brine shrimp is a crustacean conventionally classified in the Mandibulata.

Mitochondrial genes were employed because explicit evolutionary rate calibrations for particular mtDNA loci recently have appeared (Lynch 1993), and because comparative data on 16S rRNA gene sequences are available for another group of marine crabs (king and hermit crabs; Crustacea: Anomura) that in striking contrast to the horseshoe crabs has undergone exceptionally *rapid* morphological evolution (Cunningham et al. 1992; see Discussion). Sequence analyses were employed because mtDNA restriction digestion profiles proved too divergent to permit meaningful phylogenetic comparisons among the horseshoe crab species (Sugawara et al. 1988), and because in general sequence analyses reveal finer details about molecular-level changes.

Results

Newly obtained sequences for the 16S rRNA locus and the cytochrome oxidase I locus are presented in tables 1 and 2, respectively. At the 16S rRNA gene, more than 480 nucleotide positions were sequenced per individual, of which slightly more than 200 were variable among the nine newly assayed specimens. At the cytochrome oxidase locus, a total of 582 nucleotide positions per individual was scored, of which 135 were variable in the survey. Most of these latter substitutions were synonymous, with only 15 (11%) of the variable positions producing amino acid substitutions in the encoded protein.

Patterns of base-compositional bias are summarized in table 3. At the cytochrome oxidase gene, most noteworthy is a pronounced underrepresentation of G at the third positions of codons, a phenomenon noted previously for metazoan mtDNA in general (see Kornegay et al. 1993). Indeed, base frequencies at the various codon positions in horseshoe crabs, as well as the magnitudes of bias in base composition, are remarkably similar to those previously reported, for example, at the cytochrome *b* gene in mammalian species (table 3; Irwin et al. 1991). In the 16S rRNA gene sequence, there was a noticeable shortage of Cs, but this pattern again was shared by all assayed taxa (and also by the king crabs and hermit crabs previously assayed) (table 3). These observations suggest that the comparative evolutionary patterns and phylogenetic conclusions drawn in the current study will not be grossly affected by *differential* patterns of base-compositional bias across the surveyed species.

Phylogeny

16S Ribosomal RNA Locus.—At the intraspecific level, no sequence differences were observed between the specimens of either *Tachypleus tridentatus* or *Carcinoscorpius rotundicauda*; a single transition distinguished the two specimens of *T. gigas*; and six substitutions (three transitions and three transversions; genetic distance 0.013) distinguished *Limulus polyphemus* A from B. The two specimens of *L. polyphemus* came from genetically distinctive Atlantic and Gulf of Mexico populations in the southeastern United States, which previously were estimated by mtDNA restriction site analyses to differ at a mean genetic distance of about 0.02 (Saunders et al. 1986).

Sequence identities (one minus sequence differences) at the 16S gene ranged from 82%–100% among the various horseshoe crabs (table 4), and thus, fell within the range of similarities (70%–100%) that Hillis and Dixon (1991) recommend as optimal for phylogenetic analyses based on rRNA loci. However, mean sequence identities with the outgroup taxa were only 0.64 and 0.65 and are thus in a range where phylogenetic analyses normally become somewhat ambiguous because of alignment difficulties and increased likelihoods of multiple substitutions at a site. In comparisons among the various horseshoe crab species, transition : transversion ratios ranged from about 4:1 to 1:1; use of a 3:1 ratio produced a genetic distance matrix summarized in table 4.

In an exhaustive parsimony search based on the 16S data set (including *Artemia* and *Vejovis*), two minimum-length networks of equal total length (358 mutational steps) were identified. The

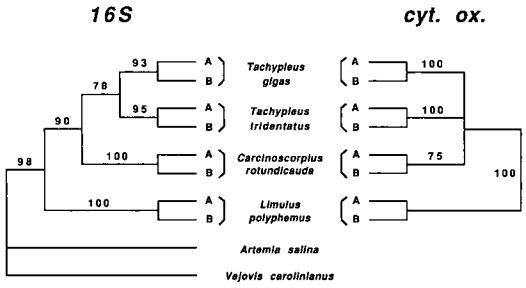

FIG. 1. Parsimony networks for horseshoe crabs based on exhaustive searches of transversional substitutions only. Left, 16S rRNA locus: minimum length phylogeny (180 steps total), with *Artemia salina* (brine shrimp) and *Vejovis carolinianus* (scorpion) as outgroups. Right, cytochrome oxidase locus: strict consensus of two minimum-length networks (58 steps each), one of which grouped *Tachypleus gigas* with *T. tridentatus* as sister taxa, and the other linked *T. gigas* with *Carcinoscorpius rotundicauda*. Also shown are levels of statistical support based on 1000 bootstrap replicates.

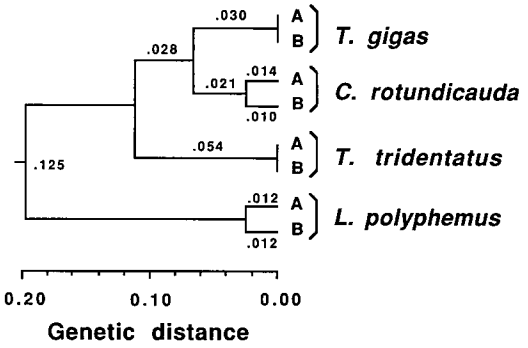

FIG. 2. UPGMA dendrogram for horseshoe crabs based on cytochrome oxidase I gene sequences from mtDNA. The genetic distance scale (method of Kimura 1980) at the bottom of the figure is drawn to indicate mean clustering levels (average sum of branch lengths connecting relevant nodes and extant taxa). Also shown on the tree are individual branch lengths from a neighbor-joining analysis that produced the same branching structure as the UPGMA dendrogram.

strict consensus of these shows the American species (*L. polyphemus*) as the sister taxon to a clade composed of the three Asiatic species, whose relationships to one another remained unresolved. In most other analyses, including consensus parsimony searches as applied to transversional substitutions only (fig. 1), and UPGMA and neighbor-joining methods as applied to the distance matrices (see fig. 4, presented later), a branching structure with *T. tridentatus* as a sister taxon to *T. gigas* was favored slightly.

Uncorrected genetic distances of horseshoe crabs to *Artemia* and to *Vejovis* appeared both large, and nearly identical (table 4). Thus, the 16S sequences of horseshoe crabs may have approached saturation with respect to base substitutional differences from the outgroups, and for this reason, we prefer to reserve judgment about the position of horseshoe crabs within Arthropoda from our data. The arrangement of *Limulus polyphemus* within the broader radiation of Arthropoda (and other invertebrate phyla) has been addressed elsewhere using nucleotide sequences from conservative nuclear rRNA loci (Field et al. 1988; Turbeville et al. 1991).

Cytochrome Oxidase Locus.—No nucleotide sequence differences were observed between the two specimens of *T. tridentatus,* nor between the two *T. gigas,* whereas 14 substitutions each (genetic distance 0.024) distinguished the conspecific samples of *C. rotundicauda,* and *L. polyphemus.* Between species, sequence identities ranged from 83% to 95%, and ratios of transition : transversion ranged from about 3:1 to 1:1 (values of 13:1 and 1:1 characterized the two sets of conspecific samples that were polymorphic, but numbers of variable sites were small). Use of a 3:1 ratio yielded the genetic distance matrix in table 4.

In an exhaustive parsimony search of the total cytochrome oxidase data, one minimum-length network (total 172 steps, seven fewer than its nearest competitor) was identified. The structure of this network is identical to that shown on the right side of figure 1, except that the "trichotomy" was resolved in favor of a sister-group relationship between *T. gigas* and *C. rotundicauda.* Parsimony analyses of first and second codon positions only, as well as UPGMA and neighbor-joining analyses of these data (fig. 2), also produced a network of this latter structure. However, parsimony analyses based solely on transversions left unresolved the exact branching order between *T. gigas, T. tridentatus,* and *C. rotundicauda* (fig. 1).

Combined Data Sets.—The 16S and cytochrome oxidase data also were combined for further parsimony analyses. When all data were considered, the aforementioned "trichotomy" among Asiatic species was resolved in favor of a *T. gigas–C. rotundicauda* clade (with bootstrap support 100%). However, based on transversions alone, *T. gigas* grouped with *T. tridentatus* (at bootstrap level 76%).

TABLE 1. Aligned sequences in horseshoe crabs of the mitochondrial gene encoding 16S rRNA. Dots indicate nucleotide identity to *Tachypleus tridentatus* (top row), and dashes indicate gaps. The gaps at positions 300 and 394 arose because previously published sequences from an outgroup, *Artemia salina* (Cunningham et al. 1992) were also included in the alignment.

	1	2 1	4 1	6 1	8 1
T. tridentatus A, B	aaaaatctgatctgcccaat	gatgaaaaattaaatggccg	cggtatattgaccgtgcgaa	ggtagcataatcatttgctt	tttaattgaaggctggaatg
T. gigas A	.....g.....	...aggt.....	.....	.....	.....g.....a.....
T. gigas B	.....g.....	...aggt.....	.....	.....	.....g.....a.....
C. rotundicauda A, B	.....a.....	...t..g....g.....	.....	.....	.....g.....
L. polyphemus A	.....ga.c.....	..g..g--...t.....	.....	.....	.....g....a.t...
L. polyphemus B	.....g..c.....	..g..g--...t.....	.....	.....	.....g....a.t...
(*V. carolinianus*)	..c.g..aagc..tgt...c	ac.acgg---.....a....	.....ta.....a..a..	.....tc.	.....g.a..a.....

	1 0 1	1 2 1	1 4 1	1 6 1	1 8 1
T. tridentatus A, B	aatggttggacgaaaaaa-g	tctgtcttagttttagtttt	taaagtttacttttagtga	aaaggctaaaattttactaa	gggacgagaagaccctatta
T. gigas A	....a.....g.....-.	.....a.....aa...	....t.....	.....tc..	.....
T. gigas B	....a.....g.....-.	.....a.....aa...	....t.....	.....tc..	.....
C. rotundicauda A, B	....a..t....g-....g.	.....a.....ag...	.t.....	g.....g...tc..	.....
L. polyphemus A	.....t.....aa	.....g.aaa...a...	.g..t....t.....	...a...g.....	.....
L. polyphemus B	.....t.....aa	.....g.aaa...a...	.g..t....t.....	...a...g.....	.....
(*V. carolinianus*)	..a....a...-t.tgt..t	.tg.....taa.a.ta...	gg..a....ta.....a.	...a.....aagtt...	a.acga.....c.

	2 0 1	2 2 1	2 4 1	2 6 1	2 8 1
T. tridentatus A, B	agctttatttttgt-ta-tt	tt-------ttattatattt	ttcattataaaaaataagag	gaattttactggggcggtag	agaaagaaaaaaatctttt-
T. gigas A	.....-.tg..	..-------.....g...	...g.....gga..	.....	.....g.g.....-
T. gigas B	.....-.tg..	..-------.....g...	...g.....gga..	.....	.....g.g.....-
C. rotundicauda A, B	.....a.tatt..	.g-------g.t....g...	..at...c.....gt.a	a.....	.....g...g.....-
L. polyphemus A	.....c....t.ggtt..	..-------.....at...	cggt.ggg...ggc.....-	..g.....	.....ttt.....-
L. polyphemus B	.....c....t.ggtt..	..-------.....at...	.ggt.ggg...ggc.....-	..ga.....	.....ctt.....a.-
(*V. carolinianus*)	..t....caa.aa.tatc..	a.tttcaaa...g..ag...	a.tg..tggctggggc..ca	a.taaa..t.ttttatt.ta	.t.ttatgg..t..ggccc-

TABLE 1. Continued.

	3 0 1	3 2 1	3 4 1	3 6 1	3 8 1
T. tridentatus A, B	---tttagaagttaaaacat	ttatgtaaatttttgatcca	tttttaatgataataagaaa	gagttactatagggataaca	gcgtaatttttc-ggagag
T. gigas A	---.....a.....	.....	..a.....g.....g	.....	.....-.....
T. gigas B	---.....a.....	.....	..a.....g.....g	a.....	.....-.....
C. rotundicauda A, B	---....a..a.....	.....t.....	..a.....a.....g	a.....	.....-.....
L. polyphemus A	---...tt.taa...t..t.	a.t...g.....c...	c.....g..g.....g..g.	a.....g.....	.....-.....
L. polyphemus B	---...tt.taa...t..t.	a.t...g.....c...	c.....g..g.....g..gt	a.....g.....	.....-.....
(*V. carolinianus*)	---..g.a..a.c....a..	..t.----------	----------...g.....g	a..aa...g.t....gg...	..a.....t-a.....

	4 0 1	4 2 1	4 4 1	4 6 1	4 8 1
T. tridentatus A, B	ttcatatcggcgaagaagtt	tgcg-acctcgatgttgaat	taaagagtcaatagggcgga	gaagttctaaatgagggtct	gttc
T. gigas A	.....	....-.....	...ga.....	.....	....
T. gigas B	.....	....-.....	...ga.....	.....	....
C. rotundicauda A, B	.....	....-.....	.....g..a.....	.....t..t.....	....
L. polyphemus A	...t.....	....-.....	.....g.....	.....tg..ga.....	....
L. polyphemus B	...t.....	....-.....	.....g.....	.....tg..ga.....	....
(*V. carolinianus*)	ga.t.c..ac.cccc...a.	..t.t...c.....g..cgg	g..ga----------	----------	----

TABLE 2. Aligned sequences of the cytochrome oxidase gene in horseshoe crabs. Dots indicate nucleotide identity to *Tachypleus tridentatus* (top row).

	1									30										60										
T. tridentatus A, B	ggc	cac	cct	gaa	gtt	tat	att	tta	att	ctc	cca	gga	ttt	gga	ata	att	tct	cat	att	att	agc	cac	caa	aca	gga	aaa	aaa
T. gigas A, B	..t	...	...	...	...	...	...	c.t	..c	..t	...	...	...	...	...	...	...	...	...	..t	...	...	...	...	...	...	...
C. rotundicauda A	..a	...	...	...	..c	...	...	c..	..c	...	...	...	...	...	...	...	...	..c	...	..t	...	...	...	...	...	...	...
C. rotundicauda B	..a	...	...	...	..c	...	...	c..	..c	...	...	...	...	...	...	...	...	..c	...	..t	...	...	...	...	...	...	...
L. polyphemus A	..g	..t	...	...	..c	..c	...	...	...	..t	..t	...	...	..g	...	..c	...	...	...	...	...	...	...	...	...	...	..g
L. polyphemus B	..g	...	...	...	..c	..c	...	...	g..	..t	..t	..g	...	...	...	..c	...	...	...	...	...	...	...	...	...	...	..g

	90							120							150												
T. tridentatus A, B	gaa	cct	ttg	gga	act	cta	gga	ata	att	tac	gct	ata	tta	gcc	att	gga	att	tta	gga	ttt	ata	gtt	tga	gct	cat	cat	ata
T. gigas A, B	...	..c	..t	...	...	t..	...	...	...	..t	...	...	c..	..a	...	...	...	c..	...	...	g..	...	...	..c	..c	...	...
C. rotundicauda A	...	..c	..t	...	t..	a..	...	...	...	..t	...	...	...	..t	...	...	...	c..	...	...	g..	...	...	..c	..c	..c	...
C. rotundicauda B	...	..c	..t	...	...	t..	...	...	...	..t	...	...	...	..a	...	...	...	c..	...	...	g..	...	...	..c	..c	..c	...
L. polyphemus A	...	...	..c	..g	...	..t	...	...	...	...	..c	...	...	..t	...	..t	...	c.t	..t	...	...	..a	...	...	..c	...	...
L. polyphemus B	...	...	..c	..g	...	..t	...	...	...	..t	..c	...	...	..t	...	..c	...	c.t	..t	...	...	..a	...	...	..c	...	...

	180														210												240
T. tridentatus A, B	ttt	aca	gta	ggc	ata	gat	gtt	gat	aca	cga	gct	tac	ttt	act	gca	gct	act	ata	att	atc	gct	gtt	cca	acc	ggt	att	aaa
T. gigas A, B	..c	...	...	...	...	...	...	...	...	...	...	..t	..c	..a	...	...	...	...	..c	..t	...	..a	...	..t	...	...	...
C. rotundicauda A	..c	...	...	...	...	...	...	...	...	...	..c	..t	..c	..a	...	...	..c	...	..c	..t	...	..c	...	...	...	...	...
C. rotundicauda B	..c	...	...	...	...	...	...	...	...	...	..c	..t	..c	..a	...	...	..c	...	..c	..t	...	..c	...	...	...	...	...
L. polyphemus A	...	...	..g	..a	...	..c	..a	..c	...	...	..a	...	..c	..a	...	...	..a	...	..c	..t	...	...	..c	..a	..a	...	...
L. polyphemus B	...	...	..g	..a	...	..c	..a	..c	...	...	..a	...	..c	..a	...	...	..a	...	..c	..t	...	...	..c	..a	..a	...	...

	270														300												
T. tridentatus A, B	att	ttt	aga	tga	cta	gct	act	tta	cat	ggc	tct	caa	atc	tct	tat	gaa	cct	cct	tta	tta	tga	gcc	tta	gga	ttt	gta	ttt
T. gigas A, B	..c	...	...	...	...	...	...	c..	...	...	...	...	..t	...	...	...	...	...	c.t	...	...	..t	...	...	...	.g.	..c
C. rotundicauda A	..c	..c	...	...	...	...	...	c..	...	...	...	...	...	...	...	...	...	..a	c.t	...	...	...	...	...	...	...	..c
C. rotundicauda B	..c	..c	...	...	...	..a	c..	...	...	..a	...	...	..t	...	...	...	...	..a	c.t	...	...	..t	...	...	...	...	..c
L. polyphemus A	..c	...	...	...	...	..c	..c	c..	...	..a	..a	...	c..	..a	..c	..c	..a	...	c.t	c..	...	..t	c..	..g	...	..t	..c
L. polyphemus B	..c	...	...	...	...	...	..c	c..	...	..a	..a	...	c..	..a	..c	..c	..a	...	c.t	c..	...	...	c..	..g	...	..t	..c

TABLE 2. Continued.

	330	360	390
T. tridentatus A, B	cta ttt act att gga gga tta aca ggt gtt gtc cta gca aac tct tcc att gac att att ctt cat gac aca tat tac gtt
T. gigas A, B	t.. ... ...a ... ..t t.. ..c ... ..c ..t ... .c ... ...t ... ...
C. rotundicauda A	t.. ..c ... ...t t.. ..c ... ..c ..t ... ...a ... ...
C. rotundicauda B	t.. ..c ... ...t t.. ..c ... ..c ..t ... ...a ... ...
L. polyphemus A	t.. ... ..a ... ...c ..a ..a a.. ... ..t ... ..c ... .t ... ..c ..c c
L. polyphemus B	t.. ... ..a ..c ... ...t ..a ..a a.. ... ..t ... ..c ... .t ... ..c ..c ... ...c c

	420	450	480
T. tridentatus A, B	gta gca cat ttt cat tac gtt tta tca ata gga gca gta ttt gca att tta gca gga gtt acc cat tga ttt cca ctc ttt
T. gigas A, B	... ..c ... .c ..t ..c c.. ... ...a ... ..c ... ...t ...
C. rotundicauda A	..g ... .c ..c ..t ... c.. ... ...t ... .c ... ...a ...
C. rotundicauda B	..g ..c ... ..c ..c ..t ..c c.. ... ...t ... .c ... ...a ...
L. polyphemus A	... ..c ... .c ..t ..c c.c ... ...c ... .g ..c ..t ... ... t.a ..c
L. polyphemus B	... ..c ... .c ..t ..c c.c ... ...c ... .g ..c ..t ... ... t.a ..c

	510	540
T. tridentatus A, B	ttt gga act tct ata aat ccc aaa tga cta aaa att cat ttt cta att ata ttt att gga gta aat acc act ttt ttc cct
T. gigas A, B	... .c ... ... t.. ... ... ac. ... ...t ..c ... ..c ... ...
C. rotundicauda A	... .a ..c ... ... .c. ... .c ... g.. ... ...t ..c ... ..c ... ...
C. rotundicauda B	... .c ..c ... ... .c. ... .c ... ac. ... ...t ..t ..c ... ..c ... ...
L. polyphemus A	... ..g .ta g.a ... ..c ... ...c ... t.. g.. ... ...g ... ..c gt. ... ..c ... ...
L. polyphemus B	... ... .ta g.a ... ..c ..a ... ...c ... t.. g.. ... ...c gt. ... ..c ... ...

	570
T. tridentatus A, B	caa cac ttt tta ggt
T. gigas A, B	... ..t ... c.. ..a
C. rotundicauda A	... ..t ... ...
C. rotundicauda B	... ..t ... c.. ...
L. polyphemus A	... ..t ..c c.. ...
L. polyphemus B	... ..t ..c c.. ...

TABLE 3. Base composition (percentage) at the first, second, and third positions of codons in the cytochrome oxidase gene, and in the overall sequence at the 16S rRNA gene.

Species	First				Second				Third			
	G	A	T	C	G	A	T	C	G	A	T	C
Cytochrome oxidase locus												
Tachypleus tridentatus	28.9	28.9	24.7	17.5	14.4	19.1	43.3	23.2	0.5	40.7	44.8	13.9
Tachypleus gigas	29.4	28.9	23.7	18.0	14.9	19.1	42.3	23.7	0.0	41.2	43.3	15.5
Carcinoscorpius rotundicauda	29.9	28.4	23.7	18.0	14.9	19.1	43.3	22.7	0.5	41.2	37.1	21.1
Limulus polyphemus	27.8	29.0	27.3	15.0	14.4	19.1	44.3	22.2	4.6	38.7	31.4	25.2
Mean	29.0	29.0	24.9	17.1	14.6	19.1	43.3	23.0	1.4	40.5	39.2	18.9
Bias*		0.107				0.244				0.396		
(mammals, cyt *b*)†												
Mean	21.6	29.4	22.7	26.3	13.6	20.2	41.6	24.7	3.6	42.7	16.3	37.4
Bias*		0.076				0.221				0.401		

	G	A	T	C
16S rRNA locus				
Tachypleus tridentatus	21.0	33.8	34.7	10.5
Tachypleus gigas	22.2	32.7	34.6	10.5
Carcinoscorpius rotundicauda	21.3	33.2	35.3	10.2
Limulus polyphemus	24.2	29.1	35.3	11.3
Vejovis carolinianus	18.6	36.3	31.8	13.2
Mean	21.5	33.0	34.3	11.1
Bias*		0.231		
(king and hermit crabs)‡				
Mean	17.0	37.4	34.3	11.3
Bias*		0.289		

* Bias in base composition, calculated as $C = \frac{2}{3} \Sigma |c_i - 0.25|$, where c_i is the frequency of the ith base.

† Shown, for comparison, are mean values for the mitochondrial cytochrome *b* gene in 20 mammalian species (from Irwin et al. 1991).

‡ Shown, for comparison, are mean values for the 16S rRNA gene in ten species of king crabs and hermit crabs (from data in Cunningham et al. 1992).

TABLE 4. Genetic distances (top line, uncorrected for multiple hits; second line, Kimura's two-parameter correction) at the 16S rRNA (above diagonal) and cytochrome oxidase I (below diagonal) gene sequences for horseshoe crabs and outgroups. In parentheses are observed numbers of transversional differences.

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
(1) *Tachypleus tridentatus* A	0.000	0.000 0.000 (0)	0.062 0.065 (6)	0.064 0.067 (6)	0.092 0.100 (17)	0.092 0.100 (17)	0.140 0.163 (34)	0.142 0.167 (37)	0.356 0.663 (86)	0.344 0.486 (69)
(2) *T. tridentatus* B	0.000 0.000 (0)	0.000	0.062 0.065 (6)	0.064 0.067 (6)	0.092 0.100 (17)	0.092 0.100 (17)	0.140 0.163 (34)	0.142 0.167 (37)	0.356 0.663 (86)	0.344 0.486 (69)
(3) *T. gigas* A	0.101 0.110 (14)	0.101 0.110 (14)	0.000	0.002 0.002 (0)	0.088 0.095 (16)	0.088 0.095 (16)	0.167 0.199 (34)	0.167 0.200 (37)	0.351 0.613 (86)	0.354 0.475 (67)
(4) *T. gigas* B	0.101 0.110 (14)	0.101 0.110 (14)	0.000 0.000 (0)	0.000	0.085 0.093 (16)	0.085 0.093 (16)	0.165 0.196 (34)	0.165 0.198 (37)	0.349 0.607 (86)	0.352 0.470 (67)
(5) *Carcinoscorpius rotundicauda* A	0.100 0.108 (14)	0.100 0.108 (14)	0.067 0.071 (16)	0.067 0.071 (16)	0.000	0.000 0.000 (0)	0.176 0.213 (40)	0.180 0.220 (43)	0.364 0.656 (90)	0.328 0.456 (66)
(6) *C. rotundicauda* B	0.113 0.125 (16)	0.113 0.125 (16)	0.053 0.056 (14)	0.053 0.056 (14)	0.024 0.024 (8)	0.000	0.176 0.219 (40)	0.180 0.220 (43)	0.364 0.656 (90)	0.328 0.456 (66)
(7) *Limulus polyphemus* A	0.163 0.192 (35)	0.163 0.192 (35)	0.167 0.197 (37)	0.167 0.197 (37)	0.170 0.200 (35)	0.167 0.196 (35)	0.000	0.013 0.013 (3)	0.354 0.657 (96)	0.367 0.572 (77)
(8) *L. polyphemus* B	0.163 0.192 (36)	0.163 0.192 (36)	0.167 0.197 (38)	0.167 0.197 (38)	0.167 0.196 (36)	0.168 0.199 (36)	0.024 0.024 (1)	0.000	0.359 0.660 (97)	0.359 0.552 (74)
(9) *Vejovis carolinianus*	—	—	—	—	—	—	—	—	0.000	0.427 0.897 (96)
(10) *Artemia salina*	—	—	—	—	—	—	—	—	—	0.000

Discussion

Cladogenetic Branching Order

The mtDNA sequence data are consistent with the view that living horseshoe crabs constitute a rather closely knit assemblage relative to the outgroups, and that the North American species (*Limulus polyphemus*) is the sister taxon to the three Asian species. These results agree with previous conclusions based on serological studies (Shuster 1962), amino acid sequence analyses of a fibrinopeptide-like protein (Shishikura et al. 1982), immunological comparisons of hemocyanins (Sugita 1988), two-dimensional electrophoresis of general proteins (Miyazaki et al. 1987), results of interspecific hybridization experiments (Sekiguchi and Sugita 1980), and cladistic appraisals of morphological characters (Fisher 1984). These conclusions also are in accord with current taxonomy, which places *Limulus* in the subfamily Limulinae, the three other extant horseshoe crabs in Tachypleinae, and all four species of Limulidae as sole living representatives of the Merostomata (Yamasaki 1988).

Within the clade composed of the Asiatic species, a conservative interpretation is that the mtDNA data leave unresolved the phylogenetic branching order for *Tachypleus gigas, T. tridentatus,* and *Carcinoscorpius rotundicauda.* Conflicts between networks based on alternative mtDNA data bases, and between alternative procedures of data analysis, suggest that the three Asiatic species probably stem from two lineage bifurcations relatively close in time. This conclusion also can be interpreted as consistent with other lines of evidence: cladistic assessments of morphological traits tend to link *T. gigas* with *T. tridentatus* (Fisher 1984), but immunological comparisons and analyses of fibrinopeptide sequences suggest that *C. rotundicauda* and *T. tridentatus* form a clade. In theory, when phylogenetic nodes are spaced closely in evolutionary time (relative to the effective size of the populations that traversed these speciation events), the lineages of independent loci and the traits they encode may truly sort into descendant taxa in such a way as to lead to apparent discordancies in structure among separate gene genealogies (such as those provided by unlinked nuclear genes, or between nuclear genes and mtDNA), as well as to occasional discrepancies between the "consensus" organismal phylogeny and the structure of any single gene tree (such as that provided by mtDNA) (Neigel and Avise 1986; Pamilo and Nei 1988; Tajima 1983; Takahata 1989). Under this line of argumentation, weak statistical support in the genealogies for particular well-assayed loci, as well as "inconsistencies" across independent gene genealogies, themselves become prima facie criteria for recognizing approximate multichotomies in an organismal phylogeny. Both of these criteria would appear to apply to the Asiatic horseshoe crabs.

In the case of the mtDNA data (and perhaps unlike the situation for morphological characters), failure to resolve the phylogenetic branching order of the Asiatic species cannot be attributed solely to a paucity of variable characters for analysis. In the combined 16S and cytochrome oxidase data sets for all horseshoe crabs assayed, more than 230 nucleotide positions exhibited variation, and more than 130 varied within the Asiatic clade alone. Thus, the lack of phylogenetic resolution appears attributable more to evolutionary "noise" at the level of individual nucleotide positions (i.e., homoplasy and/or retentions of plesiomorphic characters) than to a lack of molecular genetic diversity.

Molecular Rates and Nodal Dates

Apart from cladistic assessments *per se,* what are the approximate dates of the speciational nodes to which the extant horseshoe crab lineages trace? Morphological and biogeographic evidence have provided only the crudest of clues. According to Fisher (1984), "there is no satisfactory control on the age of the most recent common ancestor of any of the three Indo-Pacific species," and, thus, most speculation has centered on possible separation dates between the lineages leading to extant Limulinae (North America) versus Tachypleinae (Asia). From provisional generic assignments of fossils of known age (in particular, the extinct species "*Tachypleus decheni*" and "*Limulus coffini*"), and from biogeographic arguments involving the opening of the North Atlantic Ocean, rough dates of about 75 Mya and 90 Mya, respectively, have been postulated for the Limulinae–Tachypleinae separation (Fisher 1984). In studies of fibrinopeptide amino acid sequences, Shishikura et al. (1982) postulated that the Limulinae–Tachypleinae split occurred about 135 Mya, but this appears to have been based more on fossil and/or biogeographic inference than on independent time appraisals from the molecular data themselves.

From analyses of published DNA sequences as interpreted against fossil evidence or biogeo-

TABLE 5. Separation dates among horseshoe crab lineages as estimated from previous clock calibrations for the mitochondrial 16S rRNA and cytochrome oxidase I loci.*

	Millions of years ago, from clock calibrations			
	Cunningham et al. (1992)†		Lynch (1993)‡	
Node leading to	16S rRNA	(95% CL)	16S rRNA	cyt. ox. I
Tachypleus gigas vs. *T. tridentata*	17	(6–28)	0	0
Carcinoscorpius vs. *Tachypleus*	27	(12–41)	0	15
Limulinae vs. Tachypleinae	52	(27–77)	46	59

* If rates of mtDNA evolution in horseshoe crabs are lower than these conventional estimates (as is suggested provisionally by both absolute and relative rate comparisons—see text), then divergence times would have to be adjusted backward (to older dates) accordingly.

† Confidence intervals suggested by Cunningham (pers. comm. 1994) based on a correction for nonindependence of pairwise distances for king crab–hermit crab species, and are slightly wider than the uncorrected limits implied by the original Cunningham et al. (1992) treatment.

‡ Based on equation (13) in Lynch (1993), using the following parameters as suggested by the author: for nucleotide sequences at the 16S rRNA locus, I_∞ (asymptotic genetic identity as time approaches infinity) = 0.30, δ (probability of a substitution per site per billion years) = 0.44, and H_0 (nucleotide diversity in the ancestral population) = 0.13; for amino acid sequences at the cytochrome oxidase locus, $I_\infty = 0.08$, $\delta = 0.30$, and $H_0 = 0.022$.

graphically inferred dates of evolutionary separation in other animal groups, two independent clock calibrations recently have been proposed for the mtDNA loci surveyed in this report. An empirical rate estimate for the 16S rRNA gene for crustaceans (including king crabs and hermit crabs) was proposed by Cunningham et al. (1992), and calibrations for both the 16S rRNA and cytochrome oxidase I loci across a much broader diversity of vertebrate and invertebrate taxa were proposed by Lynch (1993). These calibrations as applied to the horseshoe crab data suggest that the three Asiatic species separated from one another between 0 and 30 Mya, and that their most recent shared ancestor with *L. polyphemus* dates to approximately 45–60 Mya (table 5).

Some points should be addressed about these estimates. First, within the Asiatic clade, species divergence times under the Lynch (1993) approach are somewhat more recent than those derived from Cunningham et al.'s (1992) calibration. One factor contributing to this outcome involves Lynch's correction for mean baseline nucleotide diversity ("intraspecific" variation just prior to the time that populations would be recognizable as species). This adjustment accounts for why some of the divergence-time estimates are 0 Mya under the Lynch method. A take-home message is that divergence times inferred for recently separated species can be highly sensitive to levels of genetic variability assumed for the ancestral taxa.

A second point is that the molecular-based estimates of divergence time for the Limulinae–Tachypleinae split are somewhat more recent than suspected from the fossil and/or biogeographic evidence noted above. Does this imply that mtDNA sequence evolution in horseshoe crabs has been slower than under the calibrations of Lynch and Cunningham et al.? In the absence of reliable independent information (fossil or biogeographic) on separation dates for horseshoe crab lineages, an alternative approach involves relative rate tests against outgroups. These evaluations too pose several difficulties, stemming from: (1) absence of clear independent knowledge about which Arthropod or other taxa would constitute an appropriate and usable outgroup; (2) the possibility that phylogenetic connections of any outgroups to horseshoe crabs might be quite ancient relative to separation dates among ingroup members; and (3) the likelihood that the dynamics by which mutational differences accumulate at mtDNA (or other) loci could be strongly nonlinear over the potentially long timescales involved (caused by, e.g., mutational saturation at potentially variable nucleotide positions, or to rate heterogeneities along extended branches). With these caveats in mind, provisional tests of relative rate nonetheless can be conducted with available data.

Assume that current taxonomy correctly reflects phylogeny, such that the scorpion *Vejovis* truly is a sister taxon to the xiphosurans (relative to the brine shrimp), and that *Artemia* is therefore an appropriate outgroup. Then, following the logic of figure 3, evolution of the 16S rRNA gene in the *Vejovis* lineage appears to have been about four times greater, on average, than that leading to the extant horseshoe crabs. Alternatively, if *Vejovis* is assumed to be an outgroup to a sister-taxon clade composed of *Artemia* and

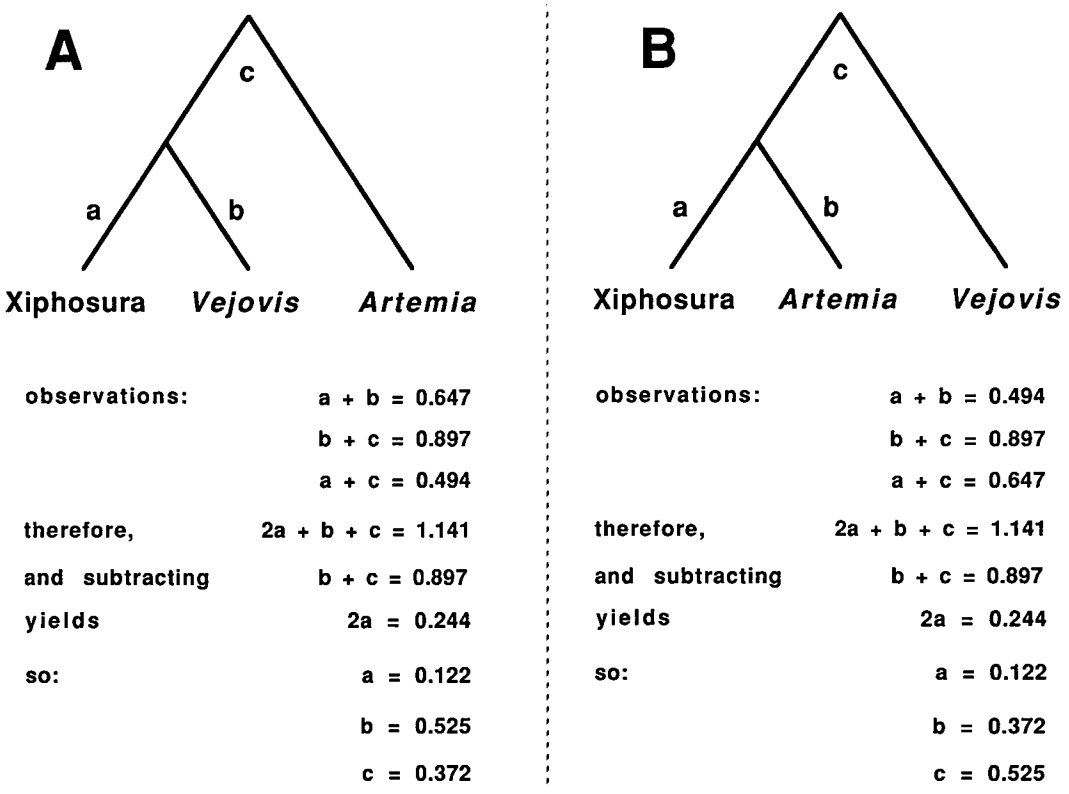

FIG. 3. Relative rate tests for 16S rRNA gene sequences in horseshoe crabs (Xiphosura) under alternative assumptions that (A) *Vejovis* is a sister taxon to the exclusion of *Artemia*; and (B) *Artemia* is a sister taxon to the exclusion of *Vejovis*. Note that in either case, the inferred branch length "a" leading to the Xiphosurans is shorter than the inferred branch length "b" leading to the sister taxon.

the xiphosurans, then by similar calculations the rate of 16S gene evolution in the *Artemia* lineage has been about three times greater than that leading to the horseshoe crabs. Similar tests of relative rate as applied to counted numbers of transversions (rather than adjusted distance estimates) yields apparent slowdowns in horsehose crab lineages by 1.2–1.9-fold. Thus, these several analyses suggest that there may have been a slowdown in the evolutionary rate of the 16S rRNA gene in horseshoe crab lineages relative to rates in these other Arthropods. If so, estimated separation dates in the xiphosuran phylogeny would have to be adjusted backward in time accordingly, relative to the dates presented in table 5.

In summary of this section, despite the availability of considerable molecular sequence information, we appear to have gained relatively little firm knowledge about the absolute dates of branch points in the horseshoe crab phylogeny. Such results imply a general irony for the field of molecular evolution—secure molecular inferences about phylogenetic nodes in sidereal time will be most difficult to obtain precisely for those evolutionary groups in which they are most needed; that is, those groups for which there is no independent fossil or biogeographic evidence against which to calibrate internally the molecular timepieces used in the dating exercise.

Comparative Morphological and Molecular Evolution

Regardless of the particular phylogenetic inferences possible from nucleotide sequences of the mtDNA genes from extant horseshoe crabs, the data also are interesting when interpreted in a comparative context against genetic distances from homologous sequences in king crabs and hermit crabs. King crabs are extremely large crustaceans with a typical crablike morphology and a strongly calcified exoskeleton, whereas the

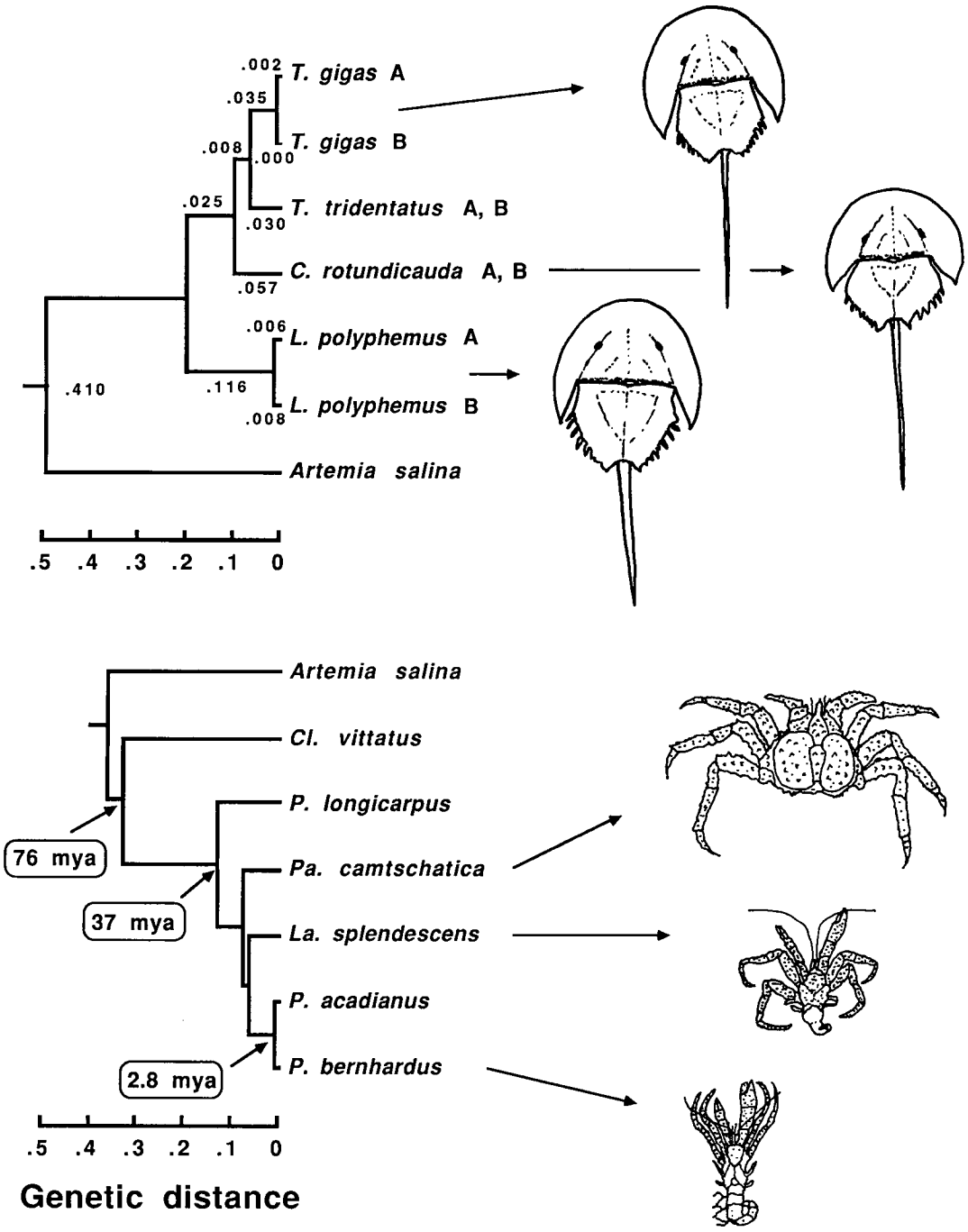

FIG. 4. Comparative UPGMA dendrograms for horseshoe crabs (above) and selected king crabs and hermit crabs (below) based on 16S rRNA gene sequences from mtDNA. The genetic distance scale (method of Kimura 1980) is the same for both phenograms, and is drawn to indicate mean clustering levels (average sum of branch lengths connecting relevant nodes and extant taxa). Also shown for the horseshoe crab lineages are individual branch lengths in a neighbor-joining tree that produced the same branching structure as the UPGMA dendrogram. Sequence data underlying the recalculated king crab/hermit crab tree are from Cunningham et al. (1992); the indicated dates for particular nodes (Mya, millions of years ago) stem from vicariant geologic evidence, and also are from Cunningham et al. (1992). *T.*, *Tachypleus*; *C.*, *Carcinoscorpius*; *L.*, *Limulus*; *Cl.*, *Clibanarius*; *P.*, *Pagurus*; *Pa.*, *Paralithodes*; *La.*, *Labidochirus*. The brine shrimp, *Artemia*, is an outgroup common to both studies.

much-smaller hermit crabs have a decalcified asymmetrical abdomen that the animals coil into adopted gastropod shells for protection. As shown by Cunningham et al. (1992) in their analysis of 165 rRNA gene sequences as interpreted against a fossil-biogeographic record, the loss of shell-living habit and the complete carcinization of king crabs from hermitlike ancestors appears to have taken place over a relatively "short" time period of 13–25 My. This "rapid" morphological shift (perhaps involving heterochrony, an evolutionary change in the timing of development; see also Gould 1992) has been accompanied by molecular differentiation at the 16S rRNA gene that if anything is somewhat lower in magnitude than that exhibited by the morphologically conservative horseshoe crabs (fig. 4). In this important sense, levels of morphological and molecular divergence are conspicuously decoupled among these arthropod lineages, a recurring theme long emphasized by evolutionists for various other organisms (e.g., King and Wilson 1975; Cherry et al. 1978).

Thus, even among the horseshoe crabs (proverbial epitomes of morphological conservatism), multitudinous nucleotide differences have accumulated among evolutionary lineages (albeit at an uncertain exact pace). Consider, for example, the American versus the Asiatic horseshoe crab species, which at face value (uncorrected for multiple hits) differed at about 16% of the total of 1066 mtDNA nucleotide positions assayed. The nuclear genome of horseshoe crabs consists of perhaps some 2.5 billion nucleotide pairs (see Galau et al. 1976). Even if nuclear genomic divergence on a per-nucleotide basis is only one-tenth that of mtDNA, the American and Asian horseshoe crabs would nonetheless differ at several tens of millions of nucleotide sites, on average. Contrast this with the small handful of morphological characters known to distinguish these species.

Our conclusions are consistent with those reached a decade ago by Schopf (1984): "Other 'living fossils,' where examined, exhibited no biochemical traits indicative of a species 'where time has stood still.' There is nothing special distinguishing the DNA or RNA of *Lingula* (Shimizu and Miura 1971a,b; Shimizu 1971), the cytochrome C of *Ginkgo* (Ramshaw et al. 1971), or the cuticle of *Peripatus* (Hackman and Golding 1975)." Although molecular rates may vary widely, a revolutionary finding would now be an example from *any* taxonomic group in which long-term evolution proved to be sharply arrested at the level of overall nucleotide sequence.

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