Tropical forests are both evolutionary cradles and museums of leaf beetle diversity

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The high extant species diversity of tropical lineages of organisms is usually portrayed as a relatively recent and rapid development or as a consequence of the gradual accumulation or preservation of species over time. These explanations have led to alternative views of tropical forests as evolutionary ''cradles'' or ''museums'' of diversity, depending on the organisms under study. However, biogeographic and fossil evidence implies that the evolutionary histories of diversification among tropical organisms may be expected to exhibit characteristics of both cradle and museum models. This possibility has not been explored in detail for any group of terrestrial tropical organisms. From an extensively sampled molecular phylogeny of herbivorous Neotropical leaf beetles in the genus *Cephaloleia*, we present evidence for (*i*) comparatively ancient Paleocene–Eocene adaptive radiation associated with global warming and Cenozoic maximum global temperatures, (*ii*) moderately ancient lineage-specific diversification coincident with the Oligocene adaptive radiation of *Cephaloleia* host plants in the genus *Heliconia*, and (*iii*) relatively recent Miocene–Pliocene diversification coincident with the collision of the Panama arc with South America and subsequent bridging of the Isthmus of Panama. These results demonstrate that, for *Cephaloleia* and perhaps other lineages of organisms, tropical forests are at the same time both evolutionary cradles and museums of diversity.

Cephaloleia | diversification | evolutionary radiation | phylogeny

The extraordinarily high species diversity of tropical forest floras and faunas is often attributed to the recent and rapid accumulation of species via high speciation rates (1–4) or the gradual accumulation and/or preservation of species over time via low extinction rates (5–8). These observations have led to the widespread belief that tropical forests are evolutionary ''cradles'' of diversity for some lineages and ''museums'' of diversity for others (6, 7).

Evidence in support of cradle models comes largely from geographic patterns of distribution, species richness, and endemism of extant tropical organisms. These patterns are often ascribed to evolutionary radiation in response to relatively recent climatic, tectonic, or biotic events; e.g., Pleistocene [≈1.8–0.01 mega-annum (Ma) ago] glaciation or Pliocene (≈5.3–1.8 Ma ago) bridging of the Isthmus of Panama (1–4). However, paleontological evidence implies that many of the evolutionary radiations that account for the present diversity (''crown diversification'') of taxonomically disparate groups of tropical organisms may have occurred comparatively early, during the late Paleocene and early to middle Eocene (Thanetian, Ypresian, and Lutetian Ages, ≈58.7–40.4 Ma ago) (9–17), associated with global warming, Cenozoic maximum global temperatures (18), and the latitudinal expansion (16, 19) and taxonomic diversification (9, 11, 14, 16, 17) of characteristically tropical lineages of plants, consistent with museum models of diversification. Although cradle and museum models are often presented as temporal alternatives (4, 7), their predictions are not mutually exclusive, and, as the aforementioned observations suggest, the evolutionary histories of tropical lineages of organisms may be expected to exhibit features of both kinds of models. However, this possibility has not been explored in detail for any group of terrestrial tropical organisms.

Using molecular genetic, paleontologic, and biogeographic evidence, we investigated timing and tempo in the diversification of *Cephaloleia*, a species-rich genus of herbivorous Neotropical leaf beetles (Chrysomelidae: Cassidinae). Our goals were to determine (*i*) whether the evolutionary history of *Cephaloleia* diversification (speciation–extinction) departs significantly from a constant rate model, (*ii*) whether *Cephaloleia* has experienced unusually rapid shifts in diversification rate during its evolutionary history, indicative of adaptive radiation(s), and (*iii*) whether, taken together, these data support temporal patterns of diversification consistent with cradle models (recent and rapid diversification), museum models (slow accumulation of diversity over time and/or preservation of comparatively ancient diversity), or some combination of timing and rate components from both kinds of models.

The genus *Cephaloleia* presents a remarkable opportunity to study timing and tempo in the taxonomic diversification of a demonstrably ancient lineage of herbivorous Neotropical insects. *Cephaloleia* is species-rich, with ≈202 extant species (20), and has figured prominently in the development of community ecological theory (e.g., ref. 21). All life stages feed on monocots, mostly in the order Zingiberales (22). A subset of species feed only in the juvenile rolled leaves of Zingiberales (Fig. 1), earning *Cephaloleia* the common name of rolled-leaf ''hispine'' beetles. *Cephaloleia*-like feeding damage on the leaves of latest Cretaceous (66.2 Ma) and early Eocene Zingiberales (20) documents the antiquity of the *Cephaloleia*/Zingiberales interaction and demonstrates that *Cephaloleia*-like beetles diverged from their most recent common ancestor before the end of the Cretaceous (65.5 Ma ago).

Results

Parsimony, maximum likelihood (ML), and Bayesian analyses of the combined mitochondrial DNA (≈1,800 bp) and nuclear DNA (≈400 bp) sequence data representing 95 *a priori* designated ingroup species, six outgroups, and 133 total specimens generated highly resolved, well supported, and compatible phylogenetic trees (see *Supporting Results and Discussion* in *Supporting Text* and Fig. 4, which are published as supporting information on the PNAS web site). *Cephaloleia* was monophyletic only with the inclusion of eight *a priori* designated *Cephaloleia*-like taxa from other genera, in agreement with ref. 22.

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Abbreviations: Ma, mega-annum; ML, maximum likelihood; PL, penalized likelihood; MCCR, Monte Carlo constant rate; LTT, lineages through time.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. DQ026066–DQ026082, DQ026084–DQ026114, DQ026116–DQ026120, DQ026122–DQ026124, DQ026126–DQ026133, DQ026135–DQ026149, DQ026151–DQ026153, DQ026155–DQ026157, DQ026159–DQ026162, DQ026165, DQ026167, and DQ538137–DQ538308).

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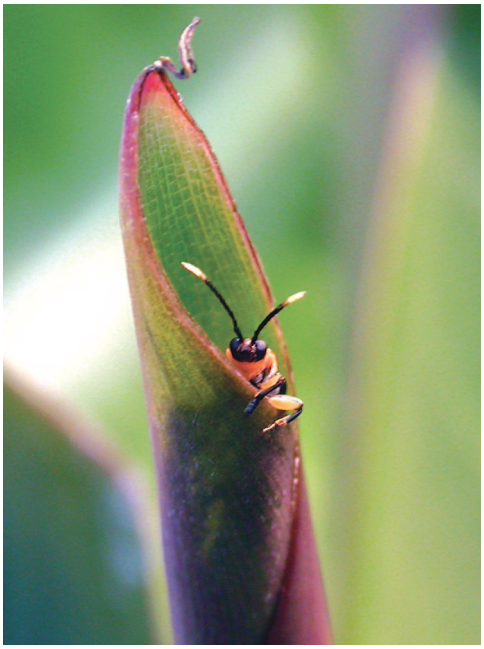

Fig. 1. *Cephaloleia variabilis* at the open tip of an immature rolled leaf of *Heliconia metallica* (Darien Province, Panama).

Interspecific ingroup *p* distances were high (mean: 16.07%), consistent with deep divergences between taxa. Intraspecific "replicates" and other identical or nearly identical sequences were excluded from further analyses (see Table 2, which is published as supporting information on the PNAS web site), leaving 83 "exemplars."

Among lineage diversification rate variation was concentrated at relatively deep nodes in the tree, as evidenced by an overall increase in *P* values for six tree balance statistics known to vary in the phylogenetic depth at which they are most sensitive (listed deepest to shallowest; $B_1 = 0.002$, $M_\Sigma = 0.003$, $M_\Sigma{}^* = 0.006$, $M_\Pi = 0.003$, $M_\Pi{}^* = 0.012$, $I_c = 0.062$) (23) (see *Supporting Materials and Methods* in *Supporting Text*). Multiple iterations of sequential deletion of taxa from rapidly evolving lineages and reanalysis did not result in significantly more balanced trees.

Based on the Monte Carlo constant rates (MCCR) test, internal nodes in the *Cephaloleia* exemplar phylogeny were significantly closer to the root than expected, indicative of a decrease in net diversification rate (speciation–extinction) over time [species (exemplars) sampled, $x = 83$; total number of species, $y \approx 202$; $\gamma = -7.82$; critical value of $\gamma = -3.77$ at $P = 0.05$]. This result was robust to taxonomic underestimation; in sensitivity analyses, values of extant species richness up to 1,523 returned a significant result. A semilogarithmic plot of lineages through time (LTT) (Fig. 2*b*) deviated significantly from a simulated curve generated under a birth–death model with incomplete taxon sampling and a constant diversification rate, corroborating evidence from the MCCR test for a decrease in diversification rate through time. Further patterns apparent in the empirical LTT plot included a trend toward reduced diversification rates beginning ≈38 Ma ago (late Eocene) and a Miocene–Pliocene increase in diversification rate that peaked ≈4–5 Ma ago. Overall, a model of diversification specifying a gradual decrease in rate through time best fit the empirical LTT plot (Figs. 2*b* and 3), consistent with results from the MCCR test.

We located unusually rapid shifts in diversification rate along branches subtending seven clades (Fig. 2*a* and Table 1). These shifts were confined to the Eocene (55.8–33.9 Ma ago; shifts 1, 2, 6, and 7) and Oligocene (≈33.9–23.0 Ma ago; shifts 3–5). No significant shifts in diversification rate were recovered in the last ≈23 Ma, perhaps because of the lack of power for these tests at shallow nodes (23). These patterns were robust to differences among alternative tree topologies. Analyses of 100 alternative ML trees and 100 randomly sampled trees from the Bayesian posterior distribution identified diversification rate shifts of comparable age and placement in the tree.

Discussion

We propose that massive alteration of ecosystems associated with the Cretaceous–Paleogene boundary event ≈65.5 Ma ago (24), including extinctions of plants and insects (25), "primed the phylogenetic fuse" for the adaptive radiation of *Cephaloleia* in the latest Paleocene–early/middle Eocene ≈54.97–43.47 Ma ago, triggered by global warming (26) and the latitudinal expansion (16, 19) and taxonomic diversification (9, 11, 14, 16, 17) of characteristically tropical lineages of plants, including important *Cephaloleia* hosts in the order Zingiberales (20, 27). The Cretaceous–Paleogene boundary event may have precipitated the extinction of many specialized plant–insect associations (25), setting the stage for later Paleocene–Eocene adaptive radiation from surviving Cretaceous lineages [such as documented here for *Cephaloleia* and elsewhere for many other groups of organisms (26)]. The proposed early Cenozoic diversification of angiosperm feeding beetle genera (28), *Phyllonorycter* moths (29), and herbivorous insects in general (10, 13) are consistent with this hypothesis. The robustness of our age estimates to relaxation of the maximum constraint on ingroup age (Table 1) lends further support to the timing of Cenozoic crown diversification reported here.

A slowdown in *Cephaloleia* diversification during the late Eocene to Oligocene (Fig. 2*b*) may have resulted from decreased origination rates [e.g., from the filling of ecological niches (30)] and/or increased extinction rates [e.g., because of the retreat of characteristically tropical lineages of plants to lower latitudes (19)]. The closely nested diversification rate shifts during the Oligocene (shifts 3–5; Fig. 2*a*; see also *Supporting Results and Discussion*) occur in a lineage composed entirely of host specialists on immature rolled leaves of plants in the genus *Heliconia* (Heliconiaceae) (22). These shifts (beginning 33.25 ± 2.91 Ma ago; Table 1) closely coincide with crown diversification of *Heliconia* beginning ≈32 Ma ago (27), possibly indicating an effect of increased niche availability. Ensuing cycles of global warming and cooling, e.g., late Oligocene and middle Miocene (see Fig. 2*b*), show little overall influence on diversification in *Cephaloleia*, perhaps because of the aforementioned prior filling of ecological niches.

We observed a slight increase in diversification rate in the LTT plot from ≈12.6 to 4 Ma ago (middle/late Miocene–early Pliocene), peaking ≈4–5 Ma ago (Figs. 2*b* and 3). This increase is coincident with the late Miocene–Pliocene collision of the Panama arc with northwestern South America ≈12.8–7.1 Ma ago (31) and subsequent bridging of the Isthmus of Panama ≈3.5 Ma ago (32). All of the cladogenetic events contributing to this peak in diversification rate (4–5 Ma ago) involve allopatric or parapatric sister species pairs, one in lower Central America and the other in extreme lower Central America and/or northwestern South America (Fig. 2*a*; see also Table 2). The near absolute allopatry or parapatry of post-Eocene (<33.9 Ma) sister species further suggests that recent speciation has occurred chiefly by vicariance, peripatry, and/or parapatry. The reconstructed evolutionary history of *Cephaloleia* diversification therefore exhibits timing and rate components characteristic of cradle and museum models, suggesting that, for *Cephaloleia* and perhaps other groups of organisms, tropical forests are at the same time both evolutionary cradles and museums of species diversity.

The relative stability of tropical environments over geological time may favor the preservation of comparatively ancient lin-

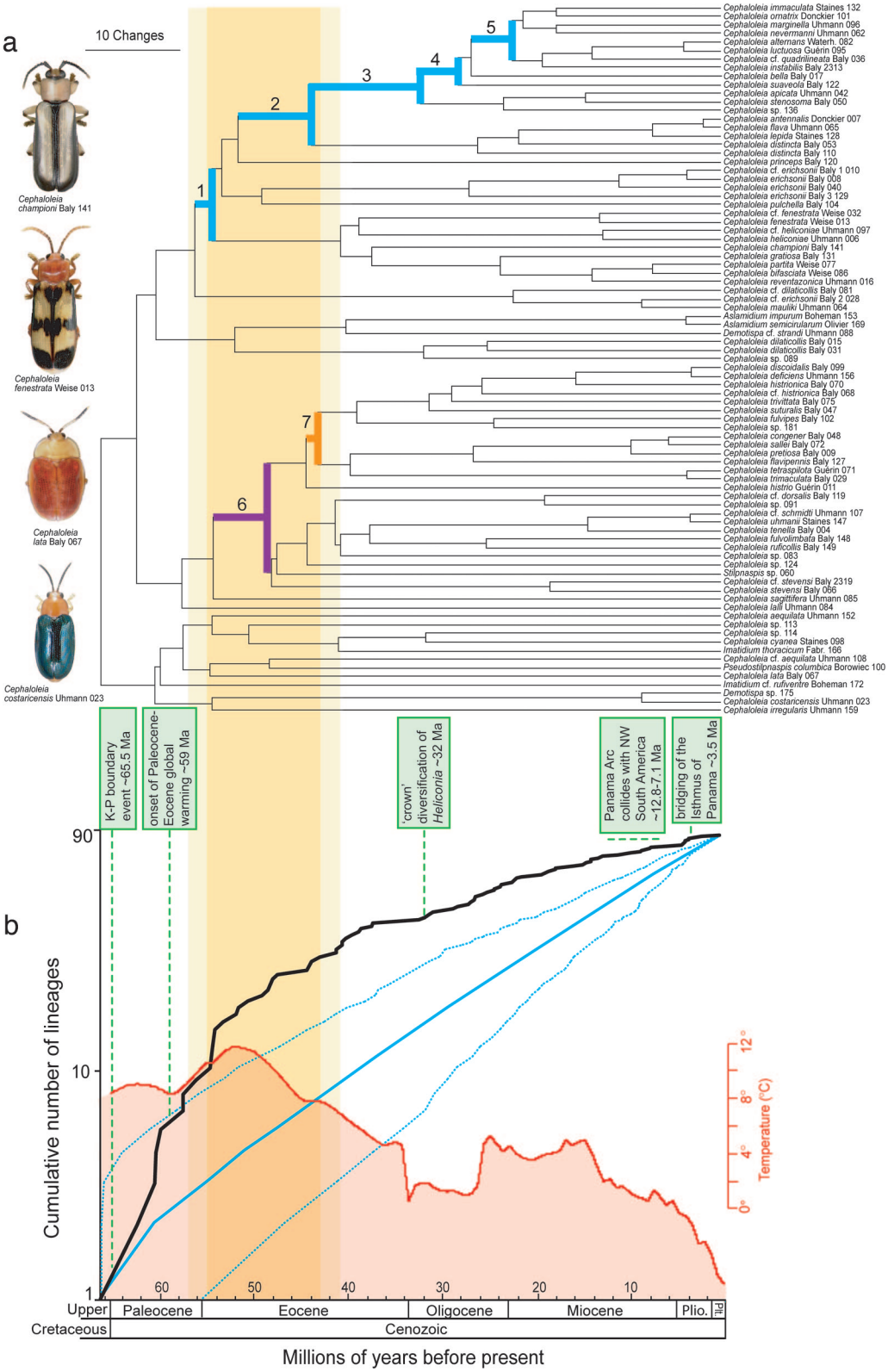

Fig. 2. Timing and tempo in the evolutionary radiation of *Cephaloleia*. (*a*) Chronogram showing significant diversification rate shifts (numbers correspond to clades identified in the text) and source(s) of support: purple (shift 6) indicates Δ_1, Δ_2, Slowinski and Guyer, and relative cladogenesis statistics; orange (shift 7) indicates Δ_1, Δ_2, and Slowinski and Guyer statistics; blue (shifts 1–5) indicates the relative cladogenesis test. Shading indicates the timeframe over which significant diversification rate shifts occurred and the corresponding outer 95% confidence interval (excluding the nested shifts 3–5, associated with crown diversification of *Heliconia*). Four specimens are shown as exemplars near their respective positions in the chronogram. (*b*) Semilogarithmic plot of LTT for *Cephaloleia* (LTT plot; black) superimposed on a time-averaged record of high-latitude sea-surface temperatures (red; adapted from ref. 18), a proxy for global climate. The constant diversification rate model is rejected if the empirical LTT curve (black) falls outside the 95% confidence interval generated by simulation (blue). Upturns or downturns in the empirical LTT plot reflect changes in rates of speciation or extinction.

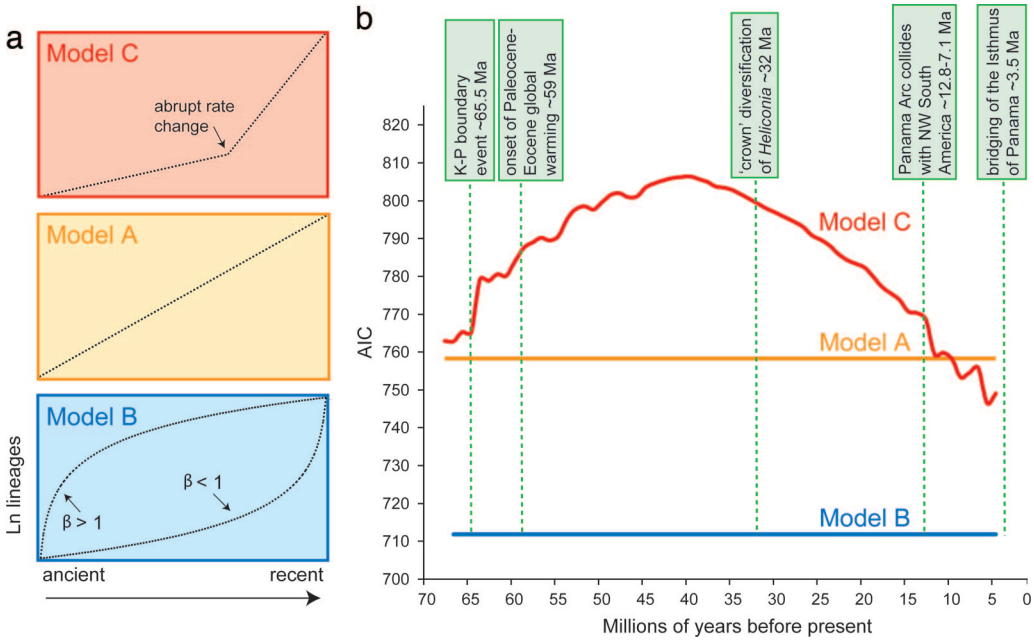

Fig. 3. Testing the fit of alternative models of diversification to the empirical LTT plot. (*a*) LTT plots illustrating the approximate pattern of diversification predicted under each of three proposed models. Model A exhibits a constant diversification rate, model B exhibits a gradually increasing ($\beta < 1$) or decreasing ($\beta > 1$) diversification rate, and model C exhibits an abrupt change in diversification rate such that two different rates best fit the data. Model C depends on the timing of the hypothesized rate shift (see *Supporting Materials and Methods*) (38). (*b*) Akaike information criteria values for models A, B, and C based on the PL chronogram. The lowest Akaike information criteria value identifies the best-fit model. Although model B is preferred over the entire evolutionary history of *Cephaloleia*, note that model C is favored over model A from ≈4 to 9 Ma ago, indicative of a shift (increase) in diversification rate but not a sufficiently abrupt shift for model C to be favored over model B. Labels identify the timing of biotic, climatic, and tectonic events identified in the text. We also tested the fit of models A and B to the empirical data using a hierarchical likelihood ratio test. Although model B provided the best fit, the likelihoods associated with models A (−378.068) and B (−353.843) were not significantly different ($\chi^2 = 48.451$, df = 1, $P = 0$).

eages resulting from adaptive radiation and facilitate the continued accumulation of species diversity, predominantly via vicariance, peripatry, and/or parapatry. Viewed in this light, major climatic, tectonic, and biotic events may be expected to leave a telltale signature on diversification histories, appropriate to the geographic/taxonomic (e.g., global, regional, or lineage-specific) and temporal scales at which they most influence diversification.

Materials and Methods

See *Supporting Materials and Methods* for more detailed methods.

Sampled Species. The species included in this study comprise a broad sample of the ecological and morphological variation known in *Cephaloleia* and potentially allied taxa in other genera (see Table 2) (33). Taking into account undescribed, cryptic, and over-split species, and with the inclusion of *Cephaloleia*-like taxa currently ascribed to other genera, we estimate that the total number of extant species in the genus is close to the 202 species reported by Wilf *et al.* (20). Outgroups included representatives of six cassidine tribes: *Alurnus ornatus* (Alurnini), *Chalepus* sp. (Chalepini), *Chelobasis bicolor* (Arescini), *Imatidium rufiventre* (Imatidiini), *Prosopodonta limbata* (Prosopodontini), *Stenispa vespertina* (Cephaloleiini), and one criocerine, *Crioceris duodeci-*

Table 1. Fossil calibrated mean estimated ages and bootstrap estimates of standard error, probabilities for the Δ_1, Δ_2, and SG statistics, and significance levels from the RC test for clades identified in the text

			P†			
Clade	Age,* Ma	Age,† Ma	Δ_1	Δ_2	SG	RC test
Ingroup	70.93 ± 3.83	66.20 ± 0.00	—	—	—	—
1	58.98 ± 4.18	54.97 ± 1.88	—	—	—	0.05
2	47.72 ± 4.38	44.48 ± 2.80	0.058	0.071	0.138	0.05
3	35.67 ± 3.85	33.25 ± 2.91	—	—	—	0.05
4	31.13 ± 3.71	28.99 ± 2.80	—	—	—	0.05
5	25.03 ± 3.42	23.30 ± 2.78	—	—	—	0.05
6	52.73 ± 3.63	49.15 ± 2.02	0.011	0.017	0.014	0.01
7	46.66 ± 3.76	43.47 ± 2.59	0.032	0.032	0.032	—

SG, Slowinski and Guyer.
*Maximum age constraint of 145.5 Ma.
†Maximum age constraint of 120 Ma.

mpunctata (tribe Criocerini). Voucher specimens are deposited with the Harvard University Museum of Comparative Zoology.

Phylogenetic Analyses. We sequenced ≈1,800 bp of mitochondrial DNA, including parts of *cytochrome oxidase I* and *cytochrome oxidase II*, *tRNA-leucine*, and an ≈400-bp fragment of the nuclear gene *elongation factor 1-α* (*EF 1-α*). We selected substitution models (MODELTEST 3.6) for use in separate and combined analyses using hierarchical likelihood ratio tests and Akaike information criteria. We used the incongruence length difference test (PAUP* 4.03b10) to assess combinability of data partitions. We used parsimony (PAUP), ML (PHYML 2.4.4), and Bayesian inference (MRBAYES 3.01) to reconstruct *Cephaloleia* phylogeny. Support for branches recovered under parsimony and ML was estimated with nonparametric bootstrap values. Decay indices (parsimony) were generated by using a command file of constraint trees (PAUP/MACCLADE 4.05). All trees were rooted with *Crioceris duodecimpunctata* (GenBank accession no. AF467886). We calculated uncorrected pairwise distances (*p* distances) (PAUP) between ingroup taxa based on the combined mitochondrial DNA and *EF 1-α*.

Tree Calibration and Dating. We used penalized likelihood (PL) (R8S 1.7) to generate an ultrametric tree (Fig. 2*a*). We used fossils to calibrate the tree and to date internal nodes, including the following. (*i*) *Cephaloleichnites strongi* (Cassidinae) feeding damage on Zingiberales leaves mapped as a minimum constraint (66.2 Ma) on ingroup age. [These fossils exhibit feeding damage from an extinct ancestor of *Cephaloleia* on the leaves of an ancient ginger, based on modern observations (20). Ginger leaves occur in even older fossil strata but have not been systematically examined for *Cephaloleia*-like feeding damage.] (*ii*) A fossil specimen of *Chalepus* sp. (Cassidinae) mapped as a minimum age constraint (44.1 Ma) to the root of *Chalepus* sp. (*iii*) A fossil specimen of *Crioceris* sp. (Criocerinae) mapped as a minimum age constraint (44.1 Ma) to the root of *Crioceris duodecimpunctata*. Monocot feeding is thought to be the pleisiomorphic condition in Cassidinae (20, 34), so we constrained the maximum age of the outgroup to 120 Ma, the approximate age of the earliest unequivocal fossil monocot (35). We separately applied a 145.5-Ma (Jurassic–Cretaceous boundary) maximum constraint on outgroup age (Table 1) because molecular clock studies suggest that monocots originated between ≈130 and 160 Ma ago (36). We used the 120-Ma constraint in all subsequent analyses. We obtained the central 95% of the distribution of age estimates for each node from the distribution of estimated ML branch lengths on the tree (generated from 100 bootstrapped data sets) under PL criteria (SEQBOOT/PHYLIP 3.5C; R8S).

Timing and Tempo of Diversification. We obtained an estimate for γ (37) from the ingroup PL chronogram (GENIE 3.0). The statistic γ indicates whether internal nodes are closer to the root or to the tips of the tree than expected under a CR model ($\gamma = 0$). A significant *P* value for a negative value of γ indicates a decrease in diversification rate over time. To account for incomplete taxon sampling, we adjusted the critical value for γ using the MCCR test (37) (MCCRTEST 1.1).

Semilogarithmic Plot of LTT. We generated an LTT plot from the ingroup PL chronogram (GENIE 3.0). Our sample of *Cephaloleia* (and allied taxa in other genera) consisted of ≈41% (83 of 202) of extant species. To evaluate the effects of incomplete taxon sampling on the slope of the empirical LTT plot, we generated 1,000 replicate phylogenetic trees with 202 extant taxa and randomly pruned 119 taxa from each tree (PHYLOGEN 1.1). These 1,000 subsampled trees with 83 taxa were then used to construct a mean LTT curve and 95% confidence interval for comparison with the empirical LTT curve. We evaluated the fit of the empirical LTT curve to each of three generalized models of diversification (38) using hierarchical likelihood ratio tests and Akaike information criteria (APE 1.8) (see Fig. 3).

Diversification Rate Shifts. To detect and locate unusually rapid diversification rate shifts and to identify putative correlates, we used four "shift" statistics differing in power and bias, two likelihood ratio-based statistics (Δ_1 and Δ_2), the Slowinski and Guyer statistic (39) (SYMMETREE 1.1), and the relative cladogenesis statistic (40) (implemented in END-EPI 1.0 as the relative cladogenesis test).

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