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Temporal lags and overlap in the diversification of weevils and flowering plants

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The extraordinary diversity of herbivorous beetles is usually attributed to coevolution with angiosperms. However, the degree and nature of contemporaneity in beetle and angiosperm diversification remain unclear. Here we present a large-scale molecular phylogeny for weevils (herbivorous beetles in the superfamily Curculionoidea), one of the most diverse lineages of insects, based on ~8 kilobases of DNA sequence data from a worldwide sample including all families and subfamilies. Estimated divergence times derived from the combined molecular and fossil data indicate diversification into most families occurred on gymnosperms in the Jurassic, beginning ~185 Ma. Subsequent colonization of early angiosperms occurred during the Early Cretaceous, but this alone evidently did not lead to an immediate and major diversification event in weevils. Comparative trends in weevil diversification and angiosperm dominance reveal that massive diversification began in the mid-Cretaceous (ca. 112.0 to 93.5 Ma), when angiosperms first rose to widespread floristic dominance. These and other evidence suggest a deep and complex history of coevolution between weevils and angiosperms, including codiversification, resource tracking, and sequential evolution.

Weevils [superfamily Curculionoidea; (Fig. 1)] are an extraordinarily successful radiation of herbivorous beetles. They reach their greatest diversity in the humid tropics, but also occur in subaquatic, subterranean, desert, tundra, and other environments at nearly all latitudes and altitudes with vegetation. Weevils collectively feed on nearly all plant taxa and all kinds of living, dead, dying, and decaying plant parts. The ~62,000 described species are classified into 7 families and ~5,800 genera. [Recent authors recognize between 6 and 22 weevil families, and between 10 and 100 subfamilies (1). Here we follow the classification of ref. 2 (see supporting information (SI) Table S1).] The likely total number of species, including those awaiting discovery or description, is conservatively estimated at more than 220,000 (2). More than 80% of living weevil species belong to the family Curculionidae, the diversity of which exceeds that of any other known family of animals (3). Today, as more than a half century ago, “the classification of Curculionidae into natural subfamilies and tribes probably remains the largest outstanding problem in the higher classification of Coleoptera” (2, 4). Consequently, relationships within Curculionidae are both of greatest interest for reconstructing the evolutionary history of weevil associations with plants, and most critical for achieving stability in weevil classification.

Weevils first appear unequivocally in the Late Jurassic fossil record (Karatau, Oxfordian-Kimmeridgian, 161.2–150.8 Ma) (5). These early weevils belong to the family Nemonychidae (2) and most likely developed in the reproductive structures of conifers in a manner similar to living nemonychids (6, 7). While early weevils most likely fed on conifers, most living weevil species are specialist herbivores on flowering plants (angiosperms; >250,000 living species). Shifts to feeding on angiosperms are associated with enhanced taxonomic diversification in weevils (6), and weevils underwent considerable diversification during the Cretaceous (145.5–65.5 Ma) (2, 5, 6), a period when angiosperms also flourished (8–10). Consequently, the extraordinary taxonomic diversity of weevils is often attributed to coevolution with angiosperms (2, 6, 11). However, because of uncertainties about higher-level relationships and divergence times in weevils, the evolutionary history of weevil-angiosperm interactions remains unclear.

To gain insight into the degree and nature of contemporaneity in weevil and angiosperm diversification, we used a large-scale temporally calibrated phylogeny for weevils estimated from combined molecular and fossil data. Our molecular data set included up to 8 kilobases (kb) of DNA sequence data (4 nuclear and 2 mitochondrial genes) from a worldwide sample of 135 weevil genera representing all families and subfamilies and 8 outgroups.

**Results**

**Weevil Relationships.** Overall, we recovered moderate to strong bootstrap (BS) or posterior probability (PP) support for ~53%
(71 out of 134) of ingroup internodes (Fig. 2). * Family-level relationships were mostly compatible with recent concepts (2, 11, 14–16); however, several new and intriguing relationships were recovered at the subfamily level. A clade including Nemognathi-dae and Anthribidae (<50% ML BS; 0.82 Bayesian PP) was sister to all other weevils. Nemognathidae was rendered paraphyletic by the family Anthribidae (minus Urodon-tinae) (<50% BS; 1.0 PP), consistent with other authors who have noted this possibility based on similarities in hind wings and other adult and larval features. Most Anthribidae use angiosperm-dependent fungi as hosts, consistent with their first appearance in the fossil record coeval with a rapidly diversifying angiosperm flora in the late Early Cretaceous (2), and much later than the largely conifer-associated Nemognathidae. Placement of the subfamily Urodon-tinae separate from other Anthribidae (and Nemognathidae) was unexpected, and conflicts with morphology. The hypothesis of monophyly for Anthribidae plus Urodontinae was rejected under BI (PP = 0), but not under ML inference (Kishino-Hasegawa test or KH, P = 0.47). The belid subfamilies Oxycoryninae (including Aglycyderini) and Belinae were recovered as sister groups within a monophyletic family Belidae (78% BS; 1.0 PP), consistent with recent analyses of morphological characters (11, 14, 16–18). We recovered moderate-to-strong support for monophyly of the families Attebellidae (<50% BS; 1.0 PP) and Caridae (100% BS; 1.0 PP) under ML and/or BI. The placement of Cardiidae as sister group of the megadactyl Brentidae plus Curculionidae received strong support under BI (54% BS; 1.0 PP), in agreement with morphology (11). The subfamilies Apioninae, Brentinae, Euryhynchinae, and Nanophyinae (but not the enigmatic Ithycerinae and Microcerinae) together comprised a monophyletic but poorly supported family Brentidae (<50% BS; 0.60 PP). The hypothesis of monophyly for Brentidae, including Microcerinae and Ithycerinae, was rejected under BI (PP = 0), but not under ML (KH, P = 0.22).

We recovered a monophyletic family Curculionidae (<50% BS; 0.61 PP; including Ithycerinae and Microcerinae). Basal positions in Curculionidae were occupied by weevils with the ancestral "pedetocal" (19) type of male genitalia: Brachycerini, Microcerinae, Platypodinae, Dryophthorinae, and Ithycerinae, followed by "higher" Curculionidae (<50% BS; 0.99 PP), comprised of groups with the derived "pedal" type of male genitalia. Brachycerinae (which in the concept adopted here also includes the Erirhiniinae of authors) occupied basal positions in Curculionidae, forming a paraphyletic grade that also included the subfamilies Dryophthorinae and Platypodinae. Ithycerinae and Microcerinae were intermingled among these "basal" cur-culionids in our analyses, in contrast with morphological studies, which increasingly favor their placement in the family Brentidae (2). The enigmatic subfamily Platypodinae (ambrosia beetles) was recovered in a position sister to Dryophthorinae (<50% BS; 0.93 PP), a relationship first proposed and supported by larval morphology (15), but not previously recovered in molecular phylogenetic studies, which mostly recover Platypodinae as apomorphic derivatives of Scolytinae. While the hypothesis of monophyly for Platypodinae plus Scolytinae was rejected under BI (PP = 0), it could not be rejected under ML (KH, P = 0.49). Dryophthorinae was monophyletic (<50% BS; 0.79 PP) minus Stenoboscini.

We recovered several groups among higher Curculionidae with moderate-to-strong internodal support (under ML or BI), comprising what are usually treated as subfamilies or tribes, e.g., Baridinae: Ceutorhynchini (97% BS, 1.0 PP), Molytinae: Lixini (100% BS, 1.0 PP), and Scolytinae (<50% BS, 0.84 PP)). However, a more thorough understanding of relationships and timing and patterns of diversification in Curculionidae will apparently require additional sampling. The limited resolution obtained herein reinforces the idea that clarifying curculionid relationships is a difficult task (2, 4, 11). Nevertheless, several groups emerge from our analyses that were suspected on the basis of similar morphological features, life histories, or habits, but whose relationships had otherwise been obscured by homoplasy.

Weevils on Plants in Evolutionary Time. We recover an Early to early Middle Jurassic origin of modern weevils (ca. Aalenian-Bathonian, 175.6–164.7 Ma), with all families and most subfamilies arising by the end of the Cretaceous (Figs. 2 and 3; Table S2). The ancestral angiosperm-associated sister groups Brentidae and Curculionidae diverged during the earlier Early Cretaceous (ca. Valanginian–Hauterivian, 140.2–130 Ma). Crown diversification of both families appears to have commenced by the late Early Cretaceous (Aptian, 125.0–112.0 Ma), and all subfamilies appeared by the early Late Cretaceous (Turonian, 93.5–89.3 Ma). The family Curculionidae shows sustained high-level diversification since then, while the family Brentidae diverged previously and later in the Cretaceous (Figs. 2 and 3). Superimposed plots of stem-group divergence times for major weevil clades, and for angiosperm dominance over the course of the Cretaceous (see Fig. 3), reveal evidence for a major increase in diversity of angiosperm-associated weevils approximately concurrent with the mid-Cretaceous rise of angiosperms to widespread floristic dominance (ca. Albian–Cenomanian, 112.0 to 93.5 Ma) (8, 9).

Discussion

The larvae and adults of most extant Nemognathidae feed on conifers, and primarily on pollen. Most Anthribidae feed on ascomycete fungi growing on or in angiosperm wood, a habit proposed to have evolved from phytophagy in decaying cones of conifers or possibly cycads (23). This scenario is compatible with our results, which support a late Cretaceous origin of Anthri-bidae (excluding Urodontinae) from within the largely conifer-associated Nemognathidae. Conifers are abundantly represented in the Mesozoic fossil record (8, 9), and are also thought to be the ancestral hosts of weevils in the families Attebellidae (2),...
Fig. 2. Maximum clade credibility tree for weevils based on the minimum age Bayesian analysis. Bayesian PP \(\geq 0.50\) and maximum likelihood BS values \(\geq 50\%\) are shown on the tree (PP/BS). Ninety-five percent confidence intervals for the ages of family- and subfamily-level clades, and for the ingroup, are indicated with blue bars. Letters correspond to fossil calibration points used in the molecular dating analysis. Numbers of described species are from ref. 2. Images of weevil exemplars are not to scale. Outgroups have been removed.
Belidae (18), and Caridae (2), consistent with estimated divergence times for stem-group representatives of these families (ca. 150–170 Ma) (see Fig. 3), which predate most estimates for the timing of first appearance of crown-group angiosperms [fossils −132–141 Ma (24), molecules −140–180 Ma (25)]. Note that Anthribidae is shown here separate from Nemonychidae (from which it is derived in our analyses), in order to accurately illustrate the disparate timing of origin and magnitude of extant diversity in these 2 groups (the species-poor anthribid subfamily Urodontinae is not shown separately from other Anthribidae). We propose that this temporal lag in the diversification of angiosperm-associated weevils is evidence for the combined major role of ecological-evolutionary opportunity and intrinsic traits (fine-tuned, elaborated, and accumulated over the course of a long history of association with living, dead, dying, and decaying plants and plant organs and tissues) in the evolutionary radiation of weevils. Patterns of weevil diversification during the time interval between the origin of each major weevil clade and the present remain unclear.

The ancestrally angiosperm-associated sister groups Brentidae and Curculionidae (2) diverged during the earlier Early Cretaceous (ca. Berriasian–Valanginian, 145.5–130 Ma). Brentidae noticeably lack associations with monocots; however, many basal Curculionidae, particularly Brachycerinae and Dryophthorinae, are associated primarily or solely with them. Monocots were among the most abundant and ecologically successful early angiosperms. Indeed, most early angiosperm fossils, for example Barremian–Aptian, are from Magnoliales or monocots (28–30), not eudicots. Most other Curculionidae, and most extant species of Anthribidae, Attelabidae, and Brentidae, feed on the living, dead, dying, or fungus-infested tissues of core eudicots, the most diverse group of living angiosperms. The large number of monocot-associated taxa near the base of the family Curculionidae is consistent with a common origin of monocot feeding. Some curculionid tribes are intimately associated with gymnosperms; however, they are all nested within clades of angiosperm-feeders. Thus, while some primitive weevils may be primary associates of gymnosperms (e.g., certain Nemonychidae, Belidae, Attelabidae, and Caridae), others, such as gymnosperm-associated Curculionidae and Brentidae, are most likely secondary colonists, an interpretation consistent with other authors (2, 11, 18, 31, 32).†

Basal Curculionidae most likely first colonized core eudicots and certain other land-plant groups that were locally abundant, unoccupied, or underexploited by other herbivores, and occurred in close ecological association with their ancestral monocy.

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†Supported by their comparatively early appearance in the fossil record (when known), Gondwanan distributions (at least of the oldest, and often conifer-associated lineages), and phylogenetic position, generally at the base of their respective groups (6).
The unusual diversity of weevils thus appears to be the result of ecological-evolutionary opportunity, combined with morphological, behavioral, and physiological (intrinsic) traits: rostrum, oviposition behavior, larval endophagy, geniculate antennae, among other life-history attributes, fine-tuned, elaborated, and accumulated over the course of a long history of association with living, dead, dying, and decaying plants and plant organs and tissues. The resulting elaborate “trophic repertoire” may have prepared or perhaps even preadapted the most speciose family Curculionidae for feeding on the morphologically, ecologically, developmentally, and biochemically diverse tissues of angiosperms. Fine-tuning and elaboration of the weevil trophic repertoire, for example conveying the ability to metabolize additional- or new-plant secondary metabolites, or to oviposit deep into plant material, not only facilitated colonization and exploitation of diverse living tissues of nearly all other kinds of landplants, but also equipped Curculionidae to adapt to and track (2, 3) the increasing complexity and diversity in chemistry, structure, growth form and habits, habitat associations, and life histories of angiosperms over the course of their evolution. A similar scenario has been proposed for leaf-mining ditrysian Lepidoptera (48), and may be expected for other insect groups (and other elements of the biota) exhibiting close ecological associations with angiosperms.

As a corollary, substantial colonization of core eudicots by Curculionidae during the Late Cretaceous and Paleogene appears only to have occurred once the weevil trophic repertoire had been suitably fine-tuned and elaborated for feeding on their diverse structures and chemistries, and once core eudicots had become sufficiently widespread and abundant to serve as suitable hosts. Other weevil families (e.g., Anthribidae, Belidae, Brentidae, Nemonychidae, and Attelabidae) also colonized angiosperms; however, they lack many of the apparent specializations present in Curculionidae for feeding on (especially living) angiosperms; therefore, they have not been as successful in colonizing these plant groups and are predominantly herbaceous, lacking the true woody tissues of eudicots. As a result, they offered rapid growth life histories, Grime’s “ruderal growth strategy” (47), and had accommodationist rather than well-defended life-history patterns for deflecting insect herbivores. Thus, monocots may have offered competition-free and plant-defense-free space relative to other early divergent groups of angiosperms. Regardless of the mechanisms invoked, monocots appear to have played a pivotal and early role in the diversification of Curculionidae.

EVALUATION
Taxon Sampling and DNA Sequencing. We analyzed up to 8 kb of DNA sequence data from a worldwide sample of 135 weevil genera representing all 7 weevil families, all 26 weevil subfamilies, and 97 genera representing most major tribes in the family Curculionidae (2) (see Tables S1 and S3). Outgroups included 7 subfamilies of basal Chrysomeloidea, the weevil sister group (6, 26), and Erichomodes sylvaticus (Cucujoidae: Protocucujidae). Six genes (2 mitochondrion and 4 nuclear) were used in this study: cytchrome oxidase I (cox I), 16S rDNA, 18S rDNA, 28S rDNA, elongation factor 1-α (EF 1-α), and arginine kinase (AK). For primers used, see Table S4.

Phylogenetic Analyses. We ran two partitioned BI phylogenetic analyses in the program BEAST 1.4.7 (65–75 million generations, 12 partitions, GTR+I+Γ substitution model, estimated base frequencies) on the maximum- and minimum-age data sets, for a total of 4 analyses. All trees were rooted with Erichomodes sylvaticus (50, 51). Graphical and statistical analyses implemented in the program Tracer 1.4 were used to assess convergence and otherwise check performance and accuracy of the BEAST analyses. Based on these results, we combined the last 5,000 trees from each of the paired minimum- and maximum-age analyses and used them to estimate PP, to obtain maximum-credibility tree estimates, and to estimate divergence times and corresponding 95% confidence intervals (LogCombiner 1.4.7, PAUP* 4.0b10, TreeAnnotator 1.4.7). We implemented a partitioned ML BS analysis (1,000 inferences, 12 partitions, CAT substitution model, individual per partition branch-length optimization) using the program RAxML 7.0.4 on the CIPRES Supercomputing Center.

Hypothesis Testing. We investigated the degree to which select alternative phylogenetic hypotheses were supported by our data by estimating the posterior probabilities of alternative topologies (under BI), and by comparing the ML trees obtained with and without monophyly constraints on each group of interest using the HK test (52) as implemented in PAUP*. 4.0310.

Divergence-Time Estimates. Divergence times were coestimated with phylogeny using the Bayesian relaxed molecular clock method (53) (BEAST 1.4.7). We assumed the uncorrelated lognormal prior model of rate change, a Yule prior process to model speciation, and used automatic tuning of operators. We conservatively selected and applied fossil age constraints from 2 recent reviews (2, 5), using only the oldest fossils that could be unequivocally assigned (based on character evidence) to extant weevil subfamilies or families in our analyses (Table S5).

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Materials and Methods
See the SI Materials and Methods for more details.