Synchronous coadaptation in an ancient case of herbivory

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Coevolution has long been considered a major force leading to the adaptive radiation and diversification of insects and plants. A fundamental aspect of coevolution is that adaptations and counteradaptations interlace in time. A discordant origin of traits long before or after the origin of the putative coevolutionary selective pressure must be attributed to other evolutionary processes. Despite the importance of this distinction to our understanding of coevolution, the macroevolutionary tempo of innovation in plant defenses and insect counterdefenses has not been documented. Molecular clocks for a lineage of chrysomelid beetles of the genus *Blepharida* and their Burseraceae hosts were independently calibrated. Results show that these plants' defenses and the insect's counterdefensive feeding traits evolved roughly in synchrony, providing macroevolutionary confirmation of synchronous plant–herbivore coadaptation. The association between these two groups of organisms was determined to be about 112 million years old, the oldest age so far for a specialized plant–herbivore association.

Coevolution, the reciprocal impact of related insect species on a group of related plants, has long been considered a major force leading to the adaptive radiation and diversification of insects and plants (1, 2). Innovations in herbivore attack and plant defense are thought to have allowed antagonists to colonize new adaptive zones, within which further evolutionary divergence occurs (3–5). A fundamental aspect of the coevolutionary process is that adaptations and counteradaptations evolve synchronously (6). Thus, if plant defensive morphology and chemistry and the feeding adaptations of insects have evolved in response to reciprocal selective pressures, a corresponding pattern of interlacing times of origin should exist (7–10). Alternatively, the evolution of purported defensive and counterdefensive traits could have been asynchronous. In this case, they could be the product of non-coevolutionary selective pressures that might even be unrelated to herbivory (10). Even though such distinctions are critical to our understanding of coevolution, the macroevolutionary tempo of innovation in plant defenses and insect counterdefenses has not been documented. Here, I use independently calibrated insect and plant molecular clocks to date an ancient interaction between a lineage of chrysomelid beetles of the genus *Blepharida* and their Burseraceae hosts. The time-scaled phylogenetic reconstructions are then used to date the origin of the interaction and of some defense innovations in these plants and the corresponding beetle counterdefenses.

In a classic pattern frequently attributed to coevolution, the related genera of Alticinae beetles *Blepharida* and *Diamphidia* feed on the sister genera *Bursera* and *Commiphora* (11, 12). The genus *Blepharida* includes >70 known species, of which roughly one-half are from tropical Africa, whereas the other half occurs in the New World tropics (13). Recent molecular phylogenetic reconstructions of *Blepharida* suggest that Neotropical *Blepharida* species constitute a monophyletic group, with Afrotropical *Blepharida* as its sister group (14).

A large proportion of New World *Blepharida* are endemic to Mexico, where they feed on the genus *Bursera* (12, 15). *Bursera* also diversified in Mexico, where >100 species exist. In Africa,

one of the main *Blepharida* hosts is *Commiphora*. *Diamphidia* from Africa and *Podontia* from Asia are also very closely related to *Blepharida* (14, 16). *Diamphidia*, the renowned poison-arrow beetle of the !Kung San of South Central Africa, also feeds on *Commiphora* (16, 17). The hosts of *Podontia* are not well known, although a few species have been found on the Anacardiaceae genus *Rhus* (13). Because *Blepharida* feeds on members of the same plant family in both the New and Old World tropics, it has been suggested that the interaction probably started before the separation of Africa and South America (15, 18).

Some *Bursera* and *Blepharida* species exhibit remarkable defensive and counterdefensive mechanisms that have been attributed to their coevolution (19, 20). In *Bursera* and *Commiphora*, terpene-containing resins are stored in reticulating networks of canals that run throughout the cortex of the stems and leaves. In some of these species, the resins are under great pressure and, when a leaf is damaged, an abundant release of resins is triggered, often as a squirt (Fig. 1). Besides being repellent and toxic, resins solidify when exposed to air and may entomb small insects completely. Yet some *Blepharida* species have developed the ability to neutralize *Bursera*'s squirt-gun defense by severing the leaf veins to stop the flow of resins before feeding on the leaves (11) (Fig. 2). An alternative defensive strategy found in other *Bursera* species is the production of complex chemical mixtures. Although most *Bursera* produce from 1 to 5 main, mostly monoterpene, compounds, some species produce complex mixtures that include between 7 and 12 mono-, sesqui-, and diterpenoids (12). Chemically complex *Bursera* species do not squirt, rely solely on their complex secondary chemistry for their defense, and are the hosts of the *Blepharida flavocostata* complex. Members of *B. flavocostata* do not cut plant canals before feeding on the leaves and, unlike all other *Blepharida* species, are able to metabolize the complex mixtures of defensive chemicals present in these hosts (11). This system has several properties that make it ideal for documentation of the macroevolutionary tempo of plant–herbivore coadaptation. First, previous ecological research has documented current intense reciprocal selection for *Bursera*'s defense syndromes and corresponding herbivore syndromes (11, 19, 20). Also, robust, multigene DNA phylogenies have recently become available for the plant and beetle genera (12, 14, 21, 22), permitting time calibration of the origin of the putative coadaptations.

Materials and Methods

Previously reconstructed molecular phylogenies of *Bursera* and *Blepharida* were used in this study (Fig. 3) (12, 14, 21, 22). *Bursera*'s phylogeny was reconstructed by using sequences from the internal transcribed spacer (ITS) region and the external transcribed spacer (ETS) region of nuclear ribosomal DNA. For species of the subgenus *Bullockia*, sequences of the 5S nontran-

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Fig. 1. Antiherbivore defense in *Bursera*. In some species, terpene-containing resins are stored under pressure in networks of canals that run throughout the cortex of stems and in the leaves. When the resin canals are punctured or severed by an insect, a high-pressure squirt of resins can be propelled as far as 2 m and drench the attacker.

scribed (5S-NTS) region were also used. The phylogeny of *Blepharida* was reconstructed by using sequences from the ITS2 region and sequences from the genes encoding cytochrome *c* oxidase subunits I and II (COI and COII) of the mitochondrial genome. The Anacardiaceae species *Spondias mombin* and the Chrysomelinae species *Timarcha tenebricosa* were used as outgroups (not shown in Fig. 3).

As a first approach to dating cladogenetic events in *Blepharida* and *Bursera*, I tested for rate heterogeneity across all species for each molecular marker by implementing likelihood ratio tests that compare the likelihood of the maximum-likelihood (ML) tree under no constraint and under the constraint of a molecular clock (23). For the mitochondrial data, the hypothesis was accepted after removing about a third of the sequences, including several that are the focus of this study. For the ITS, ITS2,

Fig. 2. Beetle counterdefensive strategy. Specialized *Blepharida* species feeding on squirting *Bursera* species can disable the squirt by severing the leaf veins where the resin canals are before feeding on the leaves. Because of the high pressure inside canals and the highly fluid nature of the resin, defusing canals is done very slowly, often taking a larva >1 h. Consuming a ''disarmed'' leaf takes between 10 and 20 min.

ETS, and 5S-NTS data sets, even eliminating many heterogeneously evolving sequences did not satisfy the assumption of rate constancy. Therefore, the penalized likelihood method (24) was implemented to estimate divergence times. This semiparametric method is analogous to smoothing methods in regression analysis and estimates unknown divergence times while smoothing the rate of change along lineages. It has proven to be effective with data sets that depart from a molecular clock, often giving better estimates than methods that rely on the clock assumption and having the advantage of not requiring elimination of sequences that evolve faster or slower than the rest (24). For each data set, a nexus file that included a tree description with branch lengths obtained with ML was used for the penalized likelihood analysis. The Q-newt algorithm was implemented for the ITS2 analysis, whereas the Powell algorithm was implemented in the COI/COII, ITS, 5S-NTS, and ETS analyses. For each of the data sets, the cross-validation option was used to find the lowest (best) cross-validation score. Smoothing values of 1, 10, 100, 300, and 1,000 were tested. The lowest (best) cross-validation scores were found for the following values of smoothing factor (S): ITS, $S = 100$; ETS, $S = 1.0$; 5S-NTS, $S = 10$; COI/COII, $S = 1.0$; ITS2, $S = 1.0$. Multiple solution points were checked with three initial starting points.

Scaling of the divergence times of the *Bursera* phylogeny with the ITS and ETS data sets was done by using biogeographic and paleontological data. A first calibration considered that the minimum age of separation of African *Commiphora* and the South American *Commiphora leptophloeos* should coincide with the breakup of West Gondwana, which was definitive for all but very capable dispersers between 95 and 100 (97.5) million years ago (Mya) (25). This calibration was based on the assumption that the distribution of *Commiphora* (most species in Africa and Madagascar, and one or two species in South America) is consistent with the hypothesis that the group originated before the major continental fragmentations of Gondwana and the separation of Africa from South America (26, 27). For the 5S-NTS sequences, the phylogeny was calibrated by using the 5–15 (10) million years (Myr) estimate for the separation of continental North American *Bursera* from peninsular *Bursera* species from Baja California (28, 29).

A second calibration of the ITS and ETS sequences involved the use of the fossil species *Bursera serrulata*, a common member of the early Oligocene (34 Myr) beds of Florissant, Colorado, and identical to the extant *Bursera tecomaca* (30). *B. serrulata* was originally described as undistinguishable from *Bursera kerberii*, a species that was at the time considered synonymous with *B. tecomaca*. Subsequent taxonomic revisions separated these two species. *B. kerberii* differs from *B. tecomaca* by having distinctive caudate leaflets. *B. tecomaca*'s leaflets are acuminate like the ones of *B. serrulata*. Thus, the date of the *B. serrulata* fossil was used to determine the minimum age of *B. tecomaca*.

A biogeographic approach similar to the one used for *Bursera* was used to date divergence times of *Blepharida*. The node of separation of African *Blepharida* and Neotropical *Blepharida* was fixed to 97.5 Myr for the ITS2 and COI/COII data sets.

Estimated ages of clade divergence according to the three markers in *Bursera* were averaged for biogeographical and paleontological calibrations. These estimates included the ages of clades that contained any highly squirting *Bursera* species, and species that have been previously characterized as having complex chemistry (11, 12). For *Blepharida* species, the two sets of estimated ages of divergence according to the ITS2 and COI/COII sequences were also averaged. Estimates of age of node divergence included clades that contain species that have the ability to disarm *Bursera*'s squirt defense and the clade of taxa that can metabolize *Bursera*'s complex chemical mixtures. Confidence limits for all estimates of time were calculated as the
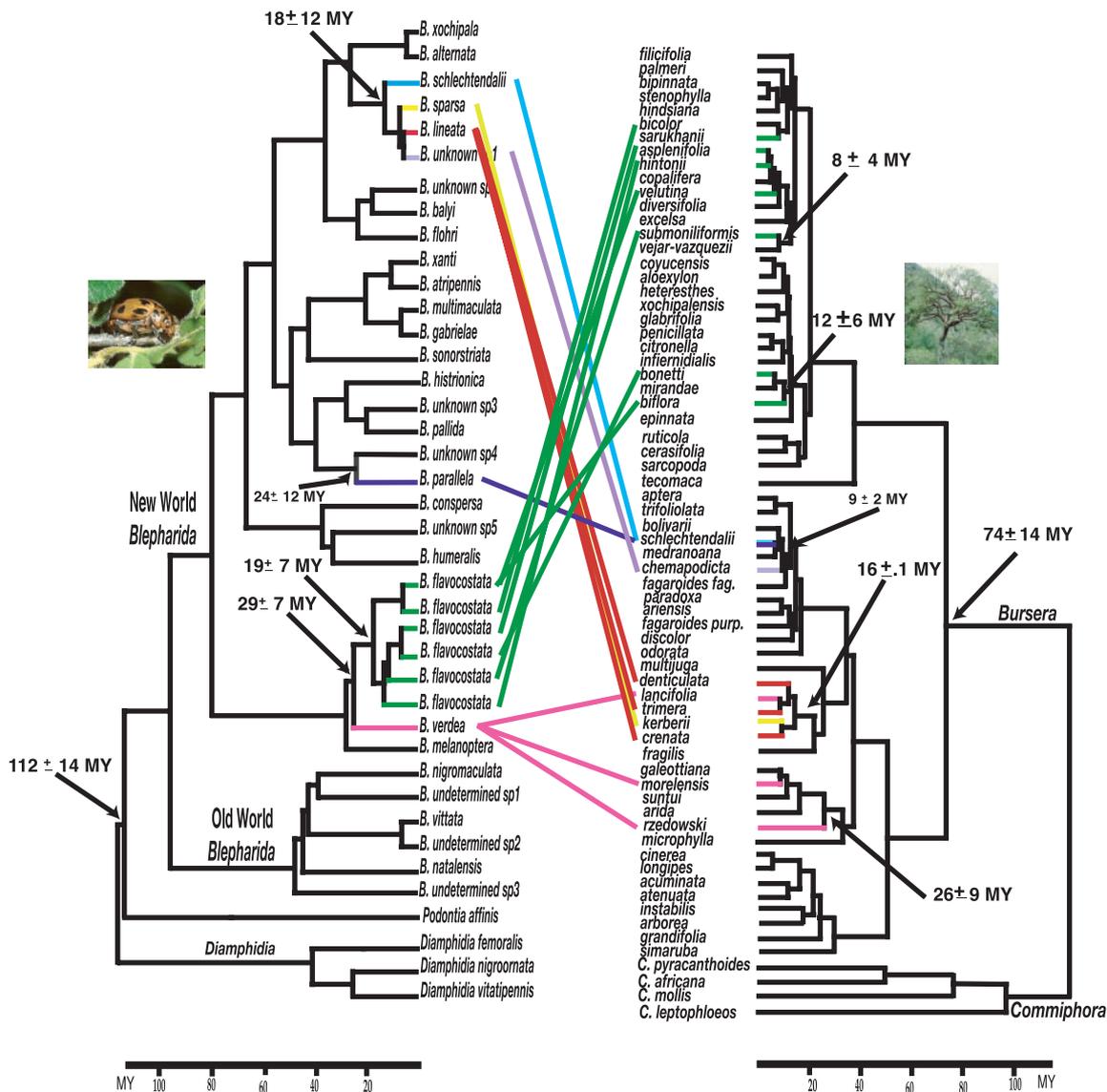
Fig. 3. Time-calibrated phylogenies of *Bursera* and *Blepharida*. Colored connecting lines indicate feeding associations of *Blepharida* on *Bursera* hosts. Colored *Bursera* species, excluding the ones in green, are highly squirting species. Colored (except green) *Blepharida* species have evolved the ability to counterattack their host's squirt defense by cutting the canals to stop the flow of resins. There are a few other squirting *Bursera* species, including *Bursera medranoana*, *Bursera arida*, and some populations of *Bursera microphylla*; however, no *Blepharida* species are known to feed on them. *Bursera* species colored in green produce chemically similar complex mixtures that include between 7 and 12 terpene compounds. Members of the *B. flavocostata* complex (colored in green) are able to metabolize the complex mixtures of defensive chemicals present in these hosts. MY, million years.

standard deviation of the clock calibrations of the different markers (31).

Results and Discussion

For *Bursera*, the biogeographic and paleontological calibrations gave very similar results. This similarity occurred because the calculated average age of divergence of *B. tecomaca* according to the biogeographic calibration of the ETS and ITS sequences (38 Myr) is very similar to the age of the fossil (34 Myr). Fig. 3 shows *Bursera*'s phylogeny time-calibrated with biogeographic information. The calibrations of the ITS, ETS, and 5S-NTS sequences also tended to give similar estimations of age of divergence, in particular for nodes with an average age of ≤35 Myr. The standard deviation of the estimates of divergence for these nodes ≤35 Myr old was between 0.3 and 9.4, with oldest divergences having the largest standard deviations. Calibrations indicate that diversification of extant *Bursera* started ≈66–74 Mya with the

division of the genus into the two subgenera. Another *Bursera* fossil species belonging to the subgenus *Bullockia*, *Bursera inaequilateralis*, is known from the flora of the Eocene Green River (32), providing independent evidence that the genus had diversified by at least 45 Mya. Calibrations also suggest that, although the genus is not new, diversification of most extant *Bursera* species is relatively recent, with many of the species originating during the past 30 Myr. This finding is concordant with ideas that the raising of the southern prolongation of the volcanic system of the Sierra Madre Occidental and the raising of the Neovolcanic axis, both starting during the Upper Oligocene, favored a warm and dry climate in the south of Mexico, propitious to the establishment of the tropical deciduous forests where the *Bursera* have diversified (33–35).

For *Blepharida*, estimates of age of divergence also had the smallest standard deviations, mostly among the most recent nodes. Values of standard errors were between 0.4 and 21 Myr.

Calibrating the separation of New and Old World *Blepharida*, with the estimate of a 95–100 (97.5) Myr-old breakup of West Gondwana, suggests that *Blepharida*'s diversification in the New World dates about 80 Mya (Fig. 3). Diversification of many extant species of New World *Blepharida* occurred during the past 30–40 Myr.

Calibrations indicate that lineages of plants that possess the defensive traits in question are of about the same age as the lineages of beetles with adaptations to counteract those defenses. Most plant lineages that include highly squirting *Bursera* species evolved in relative synchrony with the *Blepharida* lineages that include species with the ability to disarm those defenses (see colored lines, except green, in Fig. 3). For example, the lineage that includes *Blepharida schlechtendalii*, *Blepharida sparsa*, *Blepharida lineata*, and *Blepharida* unknown sp. 1 attacks some species of the lineage of squirting *Bursera* that includes *Bursera crenata*, *Bursera denticulata*, *Bursera lancifolia*, *B. kerberii*, and *Bursera trimera* (red lines in Fig. 3). These plant and herbivore lineages have diversified during the past 20 Myr. The lineage of *Blepharida verdeae*, which attacks the squirting *Bursera rzedowski*, *Bursera morelensis*, and *B. lancifolia* (pink lines), separated no later than 29 (±7) Mya, whereas its hosts diversified during the past 26 Myr.

Bursera species with complex chemistry have separated very recently, during the past 5–12 Myr. The *B. flavocostata* complex of species that attack these chemically complex species is a young lineage that has diversified in the past 5–19 Myr (see ages of taxa connected with green lines in Fig. 3). Thus, the pattern of concurrent selective stimulus and adaptive response that defines coevolution observed with the squirting plant species and vein-cutting *Blepharida* species was also observed in this chemical interaction of *Bursera* and *Blepharida* species.

The calibrated beetle phylogeny also suggests that separation of the genus *Diamphidia*, which feeds on *Commiphora*, occurred ≈112 (±14) Mya. This finding indicates that some highly specialized plant–herbivore interactions can be very old. It has often been suggested that specialization constitutes an evolutionary dead end (36, 37). However, the age of the interaction of *Diamphidia* and *Blepharida* with *Bursera* and *Commiphora* demonstrates that, rather than dead ends, highly specialized plant–herbivore associations can be maintained and even flourish over very long periods. A few other highly host-specific extant associations have been timed, such as the association of the aphid subtribe Melaphidina on sumac plants, dating probably from the early Eocene (48 Myr) (38); the one of hispine beetles on Zingiberales that is about 66 Myr old (39); and the one of *Dendroctonus* beetles on Pinaceous hosts that has persisted for the past 45 Myr (40). At >100 Myr old, the association of the *Diamphidia–Blepharida* lineage with the *Bursera–Commiphora* host lineage is the oldest tight interaction yet reported for herbivory.

Coevolution between insect herbivores and their host plants has been proposed as an important factor promoting species diversity in plants and insects. Nevertheless, until now, no examples have demonstrated synchronous reciprocal adaptation. By independently calibrating the time scale of the molecular phylogenies of *Bursera* and its herbivore *Blepharida*, I have shown that examples of ploy and counterploy have synchronous times of origin, consistent with the suggestion that these traits have evolved in response to concurrent reciprocal selective pressures. These time-scaled phylogenies have also pushed back the age of the oldest known herbivorous coevolutionary interaction to >100 Myr.

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