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27. K. B. Lim and M. Hackett, data not shown. Both *m/z* 159 and 177 lost phosphate when fragmented in the ion trap.

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Insects on Plants: Macroevolutionary Chemical Trends in Host Use

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Determining the macroevolutionary importance of plant chemistry on herbivore host shifts is critical to understanding the evolution of insect-plant interactions. Molecular phylogenies of the ancient and speciose *Blepharida* (Coleoptera)–*Bursera* (Burseraceae) system were reconstructed and terpenoid chemical profiles for the plant species obtained. Statistical analyses show that the historical patterns of host shifts strongly correspond to the patterns of host chemical similarity, indicating that plant chemistry has played a significant role in the evolution of host shifts by phytophagous insects.

What factors have directed the evolution of host shifts by phytophagous insects? This has been a central question in the field of plant-insect interactions for the last 30 years (*1*). Ehrlich and Raven (*2*) postulated that shifts to new hosts are mediated by the chemical similarity between old and new hosts and that host plant chemistry should leave its trace on phylogenetic patterns of host shifts at a macroevolutionary level. However, demonstrating a role for plant chemistry in the macroevolution of host use has been difficult (*3*). Detailed quantitative investigations have had to await the development of modern molecular and phylogenetic techniques to reconstruct accurate host and herbivore trees. Also, an evolutionary association of host shifts with plant chemistry could be spurious: Related plants have similar chemistry, and plant and herbivore phylogenies may correspond for a variety of biogeographic or ecological reasons unrelated to chemistry. In fact, some studies have shown a close correspondence of host and insect phylogenies (*4*, *5*), suggesting that the pattern of host cladogenesis may be important, and that host chemical similarity may be overemphasized. Here, a quantitative investigation of the chemical trace in the evolution of insects and their

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plant hosts is presented, using insect and plant phylogenies reconstructed from DNA sequences. These reconstructions of the ancient and diverse *Bursera-Blepharida* system were used to test the relative importance of chemical similarity and host cladogenesis in the evolutionary history of host shifts.

Burseras are the New World frankincense and myrrh. The genus comprises about 100 species distributed from the southwestern United States to Peru. It reaches its maximum diversity in the tropical dry forests of Mexico, where about 80 species occur (6). The New World members of the beetle genus *Blepharida* (Chrysomelidae: Alticinae) include about 45 species, many of them monophagous, which feed mainly on *Bursera* and a few other members of Burseraceae and its sister family Anacardiaceae (7). Although New World *Blepharida* has been combined with the Afrotropical genus *Blepharidina*, there are well-marked morphological differences between Old and New World *Blepharida*, suggesting that the New World *Blepharida* form a monophyletic group (8). The *Bursera-Blepharida* interaction is old. In the New and Old World tropics *Blepharida* feeds on Anacardiaceae and Burseraceae, suggesting that their interaction probably started before the separation of Africa and South America, more than 100 million years ago (5, 9).

Bursera produces an array of terpenes, including alpha and beta pinene, camphene, phelandrene, and limonene (10), distributed in a reticulating network of resin canals in the cortex of the stems and throughout the leaves. In some species these resins may be under considerable pressure and squirt out when leaves are damaged (11). In many plant groups terpenes are toxic or repellent to insect herbivores (12), and in *Bursera* they decrease *Blepharida* survival and growth rate (11, 13). The fact that this is an old and specialized interaction, with many species in both genera and well-known ecology and systematics, provides a valuable opportunity for testing macroevolutionary hypotheses.

The molecular phylogenies of *Bursera* and *Blepharida* (14, 15) are shown in Fig. 1. All *Blepharida* species were collected in the field directly from the species they were attacking, and their host relationships were confirmed by multiple site visits over 3 to 5 years. A dendrogram of *Bursera* species based on their chemical similarity (chemogram) was constructed to test the importance of host chemistry in host shifts (16) (Fig. 2A). For reconstruction of the evolution of the chemistry of *Bursera*, chemical classes were parsimoniously traced onto *Bursera*'s phylogeny (17). Most clades of *Bursera* include plants that are in different classes, suggesting that chemical similarity is partially independent of plant phylogeny (Fig. 2B).

To investigate whether plant chemical similarity facilitated host shifts by *Blepharida*, I compared the topology of *Blepharida*'s phylogeny with the topology of the chemogram. Also, to investigate the importance of host cladogenesis in insect shifts, I compared the topologies of the phylogenies of *Bursera* and *Blepharida*. Three techniques were used for the comparisons: character tracing, which is graphical and not statistical (17), and tree mapping (18) and Farris' distortion coefficient (19), which are statistical techniques. The latter two methods were selected because they do not depend on the operational division of clades and clusters, and their indices of similarity are sensitive even when the topologies compared are only loosely congruent, an expected scenario for phytophagous insects, which

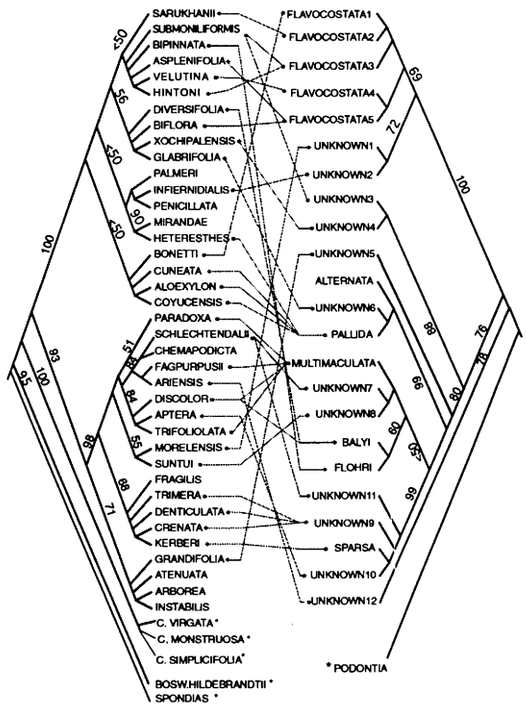

Fig. 1. Feeding associations of *Blepharida* beetles (right) on *Bursera* hosts (left). According to the molecular phylogeny of *Bursera* (strict consensus tree), the genus is monophyletic and consists of two principal groups known as sections Bullockii and Bursera. The four most parsimonious trees differed only in resolving the positions of *Bursera heteresthes*, *Bursera palmeri*, and *Bursera mirandae*. They had a consistency index of 0.57 and a retention index of 0.74. The two most parsimonious *Blepharida* trees differed in the position of *Blepharida* unknown sp. 11. They had a consistency index of 0.58 and a retention index of 0.72. Beetle species were determined by D. Furth of the Smithsonian Institution and many of them are undescribed. Sequences of insects identified as *Bursera flavocostata* present many genetic divergences and are very probably different species. For clarity, the hosts of polyphagous *Blepharida alternata* are not indicated (but see Fig. 3 for its host plants). Asterisks indicate outgroups and the numbers above the main branches of the trees are bootstrap percentages.

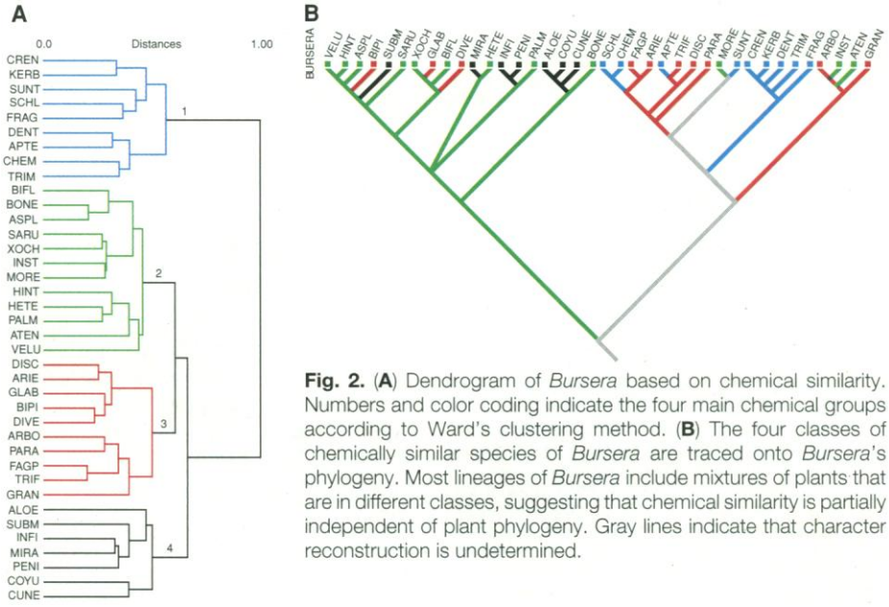

Fig. 2. (**A**) Dendrogram of *Bursera* based on chemical similarity. Numbers and color coding indicate the four main chemical groups according to Ward's clustering method. (**B**) The four classes of chemically similar species of *Bursera* are traced onto *Bursera*'s phylogeny. Most lineages of *Bursera* include mixtures of plants that are in different classes, suggesting that chemical similarity is partially independent of plant phylogeny. Gray lines indicate that character reconstruction is undetermined.

disperse rather freely among hosts. Tree mapping modifies one of two trees or cladograms until their differences are reconciled. In the present case, it modifies the plant tree by duplicating branches until a "reconciled" tree is obtained. This technique provides two measures of fit between host and associate trees. "Leaves added" is the difference between the number of nodes in the insect and reconciled tree, and "losses" is the number of instances in which an insect species is absent where it is predicted to occur on the reconciled tree. Both parameters decrease with increasing similarities of plant and insect trees [see (*18*) for details]. Farris' distortion coefficient provides a measure of the discordance of the branching topology of two trees A and B by estimating how distorted each clade of A is on B. For each monophyletic cluster of A, one counts how many times the cluster is fragmented on the B tree. This number is divided by the number of taxa of the cluster minus one. For example, if a cluster of A includes three taxa, and they are all separate in tree B, then the coefficient for that cluster of A is 2/2 = 1. The distortion coefficient is the average of the values for all clusters of A. Perfect congruence yields a coefficient of 0, and complete distortion, a value of 1. Both approaches were tested statistically by comparing observed indices to the distribution of indices obtained by repeatedly randomizing one of the trees (Markovian model).

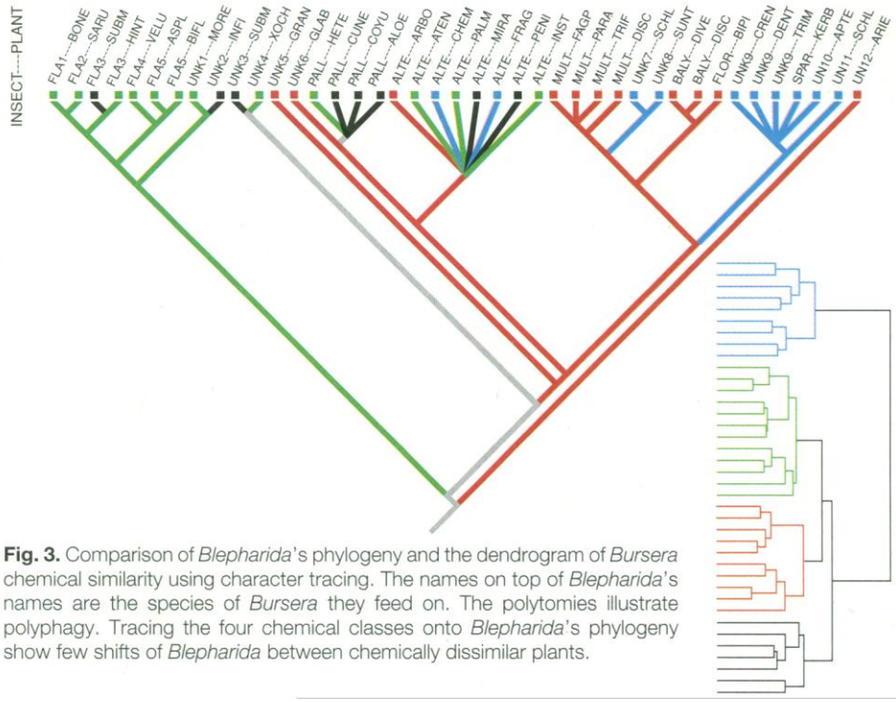

Fig. 3. Comparison of *Blepharida*'s phylogeny and the dendrogram of *Bursera* chemical similarity using character tracing. The names on top of *Blepharida*'s names are the species of *Bursera* they feed on. The polytomies illustrate polyphagy. Tracing the four chemical classes onto *Blepharida*'s phylogeny show few shifts of *Blepharida* between chemically dissimilar plants.

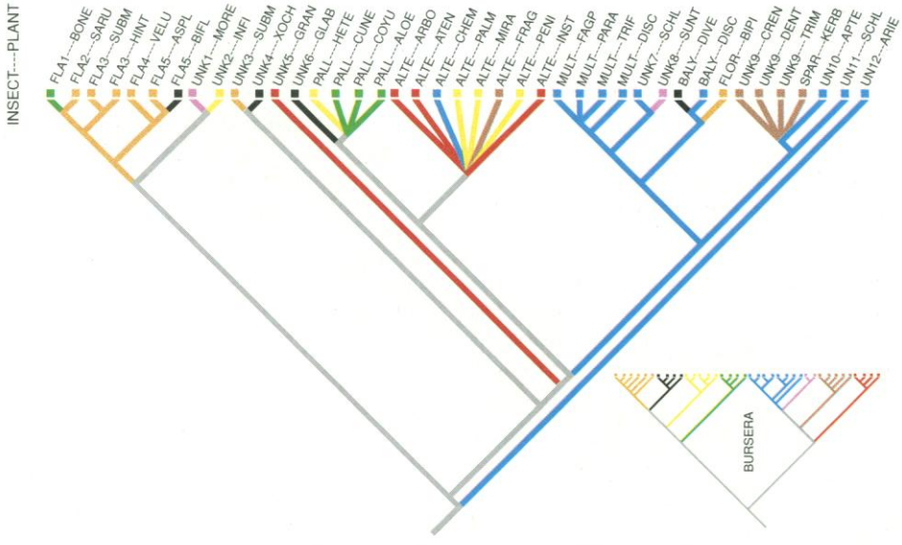

Fig. 4. Comparison of *Blepharida* and *Bursera* phylogenies. The eight major clades of *Bursera* are traced onto *Blepharida*'s phylogeny. For analyses, *Bursera mirandae* and *Bursera heteresthes* were situated according to two of the four maximally parsimonious trees. *Blepharida* has shifted numerous times among different *Bursera* lineages.

Tracing the chemical classes onto *Blepharida*'s phylogeny shows few shifts of *Blepharida* between chemically dissimilar plants (Fig. 3). Subclades of *Blepharida* appear to have colonized species of chemically similar plants. For example, the lineage that includes *Blepharida sparsa* diversified using burseras that belong only to one chemical group (in blue). Similarly, the lineage of *Blepharida flavocostata* and *Blepharida* unknown spp. 1 and 2 evolved exploiting burseras from only two chemical groups. An interesting exception is the highly polyphagous *Blepharida alternata*, which can feed on burseras from all the chemical groups. The congruence is significant with tree mapping ("leaves added," $P < 0.006$; "losses," $P < 0.0002$) and the distortion coefficient (0.73, $P < 0.05$), which do not depend on the operational delimitation of chemical classes used in the figures. Character tracing graphically demonstrates that *Blepharida* has shifted host use from one of the two major clades of *Bursera* (the two sections of the plant genus) to the other several times. *Blepharida* has also shifted between hosts belonging to different subclades several times (Fig. 4). For example, the lineage of *B. flavocostata* and *Blepharida* unknown spp. 1 and 2 attacks burseras from four terminal clades, and one clade is fairly distantly related to the others (pink line). With tree mapping, the congruence of the two phylogenies is significant for "leaves added" ($P < 0.05$), but not for "losses" ($P < 0.26$). The distortion coefficient for *Bursera* and *Blepharida* cladograms is 0.86 and congruence is not significant ($P = 0.1$).

Because chemical similarity in *Bursera* is partially independent of its phylogeny (Fig. 2B), it was possible to look at host shifts among plant chemical groups that were not host shifts among plant clades. In the same way, host shifts among plant clades that were also host shifts among chemical groups could be ignored. To do this, I modified the distortion coefficient. As mentioned before, the disagreement between trees A and B is measured by the number of fragments into which each cluster of tree A is broken on tree B. The same applies to trees A and C. But now, to measure the distortion between tree A

and tree B, for each cluster of A, the value of the distortion coefficient between A and C is added to the value of the distortion coefficient between A and B. This mathematical procedure removes shifts among chemical groups that are also shifts among plant clades. With this modification the coefficient remained statistically significant for the comparison between *Blepharida*'s phylogeny and the chemogram (0.84, $P < 0.05$). However, for the comparison of *Blepharida* and *Bursera* phylogenies, the modified distortion coefficient increased to 0.94 ($P = 0.25$). This suggests that the relationship between the two phylogenies is due in large part to the correlation between plant phylogeny and plant chemistry, whereas the relationship between *Blepharida*'s phylogeny and the chemogram of *Bursera* does not depend on the correlation between plant phylogeny and plant chemistry. Thus, comparisons ignoring the correlation between plant phylogeny and plant chemical variation, as well as comparisons controlling for this correlation, indicated a greater influence of host plant chemistry than host plant phylogeny in the evolution of host use in *Blepharida* and *Bursera*.

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15. The ITS2 region was sequenced for one individual of each *Blepharida* species found feeding on each *Bursera* species in the field and one species of its sister genus *Podontia*. Alignment resulted in a matrix of 662 characters, of which 41.7% were potentially informative. Alignment and analyses of sequences followed the same strategy as with *Bursera*.
16. Leaves of 38 *Bursera* species were collected in the field at the same time that *Blepharida* beetles were collected, and their chemical constituents were immediately extracted in ethyl acetate. Extracts were analyzed by gas chromatography, which distinguished between 10 and 15 main compounds in each species. A matrix of Euclidean distances between these species was constructed on the basis of the presence or absence of each compound. The robustness of the clusters produced was determined by looking at the consensus of three clustering techniques {Complete linkage, UPGMA, and Ward's method [P. H. Sneath and R. R. Sokal, *Numerical Taxonomy* (Freeman, San Francisco, CA, 1973); SAS Institute Incorporated, *SAS/STAT User's Guide, Version 6* (Cary, NC, ed. 4, 1989), vol. 4]}. Two of these methods agreed in dividing species into four main clusters, whereas the other (complete linkage) divided them into five by splitting cluster 4. Hewlett-Packard 5890 gas chromatograph with flame ionization detector and a 15-m column of 0.32-mm internal diameter fused silica capillary column (J & W Scientific) coated with 0.25-μm DB-5 were used for chemical analyses. Nitrogen served as the carrier gas with a linear velocity of 20.8 cm/s at a pressure of 20 kPa. Injections were made in the splitless mode with the injector at 200°C and the detector at 220°C. The oven temperature was programmed at 60°C for 1 min, then an increase of 10°C/min to 220°C, holding at 220°C for 3 min.
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Phylogenetic Analysis of Glycolytic Enzyme Expression

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Although differences among species in enzyme maximal activity or concentration are often interpreted as adaptive and important for regulating metabolism, these differences may simply reflect phylogenetic divergence. Phylogenetic analysis of the expression of the glycolytic enzymes among 15 taxa of a North American fish genus (*Fundulus*) indicated that most variation in enzyme concentration is due to evolutionary distance and may be nonadaptive. However, three enzymes' maximal activities covary with environmental temperature and have adaptive value. Additionally, two pairs of enzymes covary, indicating coevolution. Thus, metabolic flux may be modulated by many different enzymes rather than by a single rate-limiting enzyme.

Phylogenetic analyses can test for the adaptive importance of enzyme variation and address the debate concerning the control of metabolism. Many models concerning metabolic regulation have been proposed: from classical biochemical theories that predict one master regulatory enzyme per pathway (*1*), to metabolic control theories that argue that many enzymes can modulate flux (*2*, *3*). Experimental evidence suggests that the control of flux shifts among enzymes depending on laboratory conditions (*4*, *5*). In contrast, a phylogenetic perspective can reveal changes in enzyme amounts or activity produced by natural selection and thus are indicative of an enzyme's importance over evolutionary time. If variation in an enzyme's concentration is selectively important, then that variation must have functional consequences, such as changes in metabolic flux. Thus, phylogenetic analyses that identify patterns of adaptive variation in particular glycolytic enzymes suggest that variations in these enzymes are functionally important. Results from phylogenetic analyses can be compared to the predictions of different theories on metabolic control. Specifically, if there are a few master regulatory enzymes per pathway, and other equilibrium (*6*) enzymes

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