

8. C. M. Alpuche-Aranda, J. A. Swanson, W. P. Loomis, S. I. Miller, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10079 (1992).
9. D. M. Heithoff *et al.*, *ibid.* **94**, 934 (1997).
10. H. Takada and S. Kotani, in *Bacterial Endotoxic Lipopolysaccharides*, vol. 1, *Molecular Biochemistry and Cellular Biology*, D. C. Morrison and J. L. Ryan, Eds. (CRC Press, Boca Raton, FL, 1992), pp. 107–130.
11. Bacterial strains used in this study include: wild-type, ATCC14028s; PhoP^c, CS022 with *pho-24* (4); PhoP^r, CS015 with *phoP::Tn10d-Cam* (3); and PhoP^c-PmrA^r, JSG430 with *pho-24 pmrA::Tn10d-Tet* (7). The Mg²⁺-ethanol precipitation procedure for isolation of LPS was chosen because it is effective in extracting both smooth and rough LPS with a high degree of purity [R. P. Darveau and R. E. Hancock, *J. Bacteriol.* **155**, 831 (1983)].
12. Purified LPS carbohydrate profiles were examined as trifluoroacetic acid derivatives by GC. The following values for the ratio of the O-antigen sugar rhamnose to core sugar heptose were obtained: wild-type strain, 3.4; PhoP^c strain, 1.3; and PhoP^r strain, 6.6. This indicated that PhoP^c and PhoP^r LPS contained different amounts of O-antigen substitution relative to wild-type LPS. Similar results were also obtained when whole-cell rhamnose/3-OH C14:0 ratios were examined [K. Bryn and E. Jantzen, *J. Chromatogr.* **240**, 405 (1982)].
13. Lipid A was made by hydrolysis of LPS in 1% SDS at pH 4.5 [M. Caroff, A. Tacken, L. Szabo, *Carbohydr. Res.* **175**, 273 (1988)]. The presence of SDS is critical to obtain complete hydrolysis of LPS under less acidic pH conditions, such that unwanted hydrolysis of lipid A ester bonds is minimized.
14. M. Karas and F. Hillenkamp, *Anal. Chem.* **60**, 2299 (1988); M. Karas, D. Bachman, U. Bahr, F. Hillenkamp, *Int. J. Mass Spectrom. Ion Processes* **78**, 53 (1987).
15. J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong, C. M. Whitehouse, *Mass Spectrom. Rev.* **9**, 37 (1990); *Science* **246**, 64 (1989); M. Dole, H. L. Cox, J. Gieniec, *Adv. Chem. Ser.* **125**, 73 (1971).
16. See G. Odham and E. Stenhagen, in *Biochemical Applications of Mass Spectrometry*, G. R. Waller, Ed. (Wiley, New York, 1972), pp. 211–228, and references therein; N. J. Jensen and M. L. Gross, *Mass Spectrom. Rev.* **6**, 497 (1987), and references therein.
17. See E. Garcia-Vescovi, F. C. Soncini, E. A. Groisman, *Cell* **84**, 165 (1996). Wild-type *S. typhimurium* was grown in N-minimal media containing 8 μM MgCl₂, 38 mM glycerol, and 0.1% casamino acids.
18. M. Vaara, *J. Bacteriol.* **148**, 426 (1981); K. Nummilla, I. Kilpeläinen, U. Zahringer, M. Vaara, I. M. Helander, *Mol. Microbiol.* **16**, 271 (1995); I. M. Helander, I. Kilpeläinen, M. Vaara, *ibid.* **11**, 481 (1994).
19. R. A. Yost and R. K. Boyd, *Methods Enzymol.* **193**, 154 (1990).
20. The product ion mass spectra (MS²) showed different fragmentation in the ion trap (CAD by resonant excitation) relative to the triple-quadrupole instrument (conventional CAD). Fewer product ions were observed in the trap. To isolate *m/z* 323 in the ion trap, it was necessary to fragment the precursor ion (or ions) in the electrospray source, external to the trap itself. The pseudo MS² spectrum acquired under these “in source” CAD conditions at atmospheric pressure was similar to that generated using ~1 × 10⁻³ torr of argon gas in the Sciex triple-quadrupole instrument (Fig. 2A). Additional stages of isolation of the desired precursor followed by mass analysis (MS³, MS⁴) were then performed internally (Fig. 2, B and C); see J. C. Schwartz and I. Jardine, *Methods Enzymol.* **270**, 552 (1996); J. N. Louris *et al.*, *Anal. Chem.* **59**, 1677 (1987). For detailed descriptions of such MSⁿ experiments and the differences in MS-MS experiments performed with conventional beam-type instrumentation and quadrupole ion traps, see K. R. Jonscher and J. R. Yates III, *Anal. Biochem.* **244**, 1 (1997); J. F. J. Todd, in *Practical Aspects of Ion Trap Mass Spectrometry*, vol. 3, *Chemical, Environmental and Biomedical Applications*, R. E. March and J. F. J. Todd, Eds. (CRC Press, Boca Raton, FL, 1995), pp. 4–26.
21. It was also proposed that additional phosphorylation was taking place on a hydroxy acyl side chain, which was a possible explanation for *m/z* 323 (Fig. 2A) and the ion trap data (Fig. 2, B and C). Both 2- and 3-phosphoryl groups were synthesized separately by dissolving the respective hydroxy fatty acid (2 mg) in 1:3:2 chloroform/triethylamine/phosphorylchloride (total volume 1 ml), stirring at room temperature for 6 hours, followed by chloroform removal under dry nitrogen and addition of 500 μl of cold (5°C) water for 30 min. The finished product was extracted into chloroform and analyzed under the same conditions used for lipid A (see Fig. 2). Under electrospray conditions, 2-phosphoryl group was stable in chloroform solution and gave a single [M-H]⁻ (molecular anion) at *m/z* 323. The 3-phospho product was not stable and was observed to undergo a spontaneous net gain of two mass units, to *m/z* 325, followed by elimination of phosphate. Fragmentation of synthetic 2-phosphoryl group observed in the triple-quadrupole MS and ion trap did not support assigning such a side chain to the modified lipid A structure. However, a structure containing 4' phosphate cannot be ruled out.
22. J. E. Somerville, L. Cassiano, B. Bainbridge, M. D. Cunningham, R. P. Darveau, *J. Clin. Invest.* **97**, 359 (1996); R. P. Darveau *et al.*, *Infect. Immun.* **63**, 1311 (1995).
23. Two serum proteins are also involved in this process: LPS-binding protein (LBP) and either soluble or membrane-bound CD14 [S. D. Wright, R. A. Ramos, P. S. Tobias, R. J. Ulevitch, J. C. Mathison, *Science* **249**, 1431 (1990); E. A. Frey *et al.*, *J. Exp. Med.* **176**, 1665 (1992); J. Pugin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2744 (1993)].
24. P. A. Sieling *et al.*, *Science* **269**, 227 (1995).
25. P. I. Fields, E. A. Groisman, F. Heffron, *ibid.* **243**, 1059 (1989); S. I. Miller, W. S. Pulkkinen, M. E. Selsted, J. J. Mekalanos, *Infect. Immun.* **58**, 3706 (1990).
26. For previous electrospray negative-ion studies of the diphosphoryl form of lipid A, see S. Chan and V. N. Reinhold, *Anal. Biochem.* **218**, 63 (1994); A. K. Harrata, L. N. Domelsmith, R. B. Cole, *Biol. Mass Spectrom.* **22**, 59 (1993). For previous studies that used proton nuclear magnetic resonance (NMR) MS and positive-ion fast atom bombardment (FAB) MS, see K. Takayama, N. Qureshi, P. Mascagni, *J. Biol. Chem.* **258**, 12801 (1983); N. Qureshi, K. Takayama, D. Heller, C. Fenselau, *ibid.*, p. 12947; N. Qureshi, K. Takayama, E. Ribi, *ibid.* **257**, 11808 (1982). For a review of the biosynthesis, structure, and function of lipid A, see C. R. H. Raetz, *J. Bacteriol.* **175**, 5745 (1993). The attachment of aminoarabinose to the glucosamine dimer cannot be assigned unequivocally on the basis of our data alone. Because the bond linking aminoarabinose to 4' phosphate is highly labile, we have been unable to isolate an ion containing aminoarabinose linked to a fragment of lipid A, other than the molecular ion itself. However, there are other studies that support assigning the aminoarabinose substitution at the 4' phosphate position (18).
27. K. B. Lim and M. Hackett, data not shown. Both *m/z* 159 and 177 lost phosphate when fragmented in the ion trap.
28. We thank K. A. Walsh and L. H. Ericsson for MALDI-TOF and triple-quadrupole mass spectrometers; J. R. Yates III for the ion trap; M. Sanders and W. Loyd for assistance with the ion trap experiments; W. N. Howald for the GC-MS analyses; F. Turecek and W. L. Nelson for reviewing the MS results; M. Gelb for suggesting the synthesis scheme in (27); and J. Kowalak, H. Wang, J. Somerville, J. Eng, A. R. Dongre, and E. Carmack for their assistance. Supported by NIH grant R01 AI30479 (S.I.M.) and the School of Pharmacy and Department of Medicinal Chemistry, University of Washington (M.H.).

21 November 1996; accepted 20 February 1997

Insects on Plants: Macroevolutionary Chemical Trends in Host Use

Judith X. Becerra

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

Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ 85721, USA, and Instituto de Ecología, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510 México DF, México.

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).

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

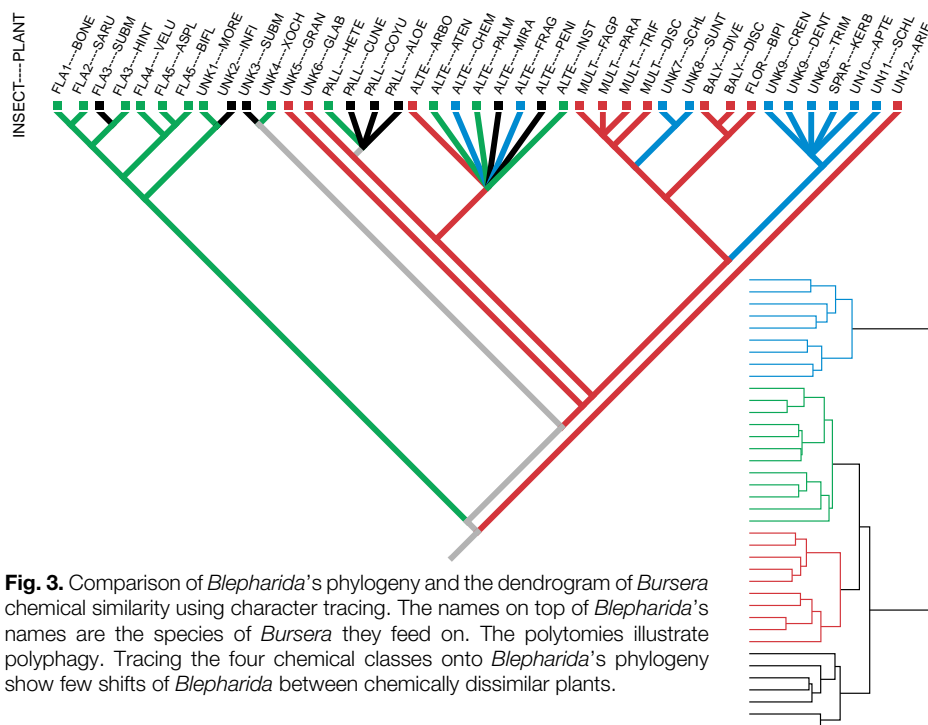


Fig. 3. Comparison of *Blepharida's* phylogeny and the dendrogram of *Bursera* chemical similarity using character tracing. The names on top of *Blepharida's* names are the species of *Bursera* they feed on. The polytomies illustrate polyphagy. Tracing the four chemical classes onto *Blepharida's* phylogeny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

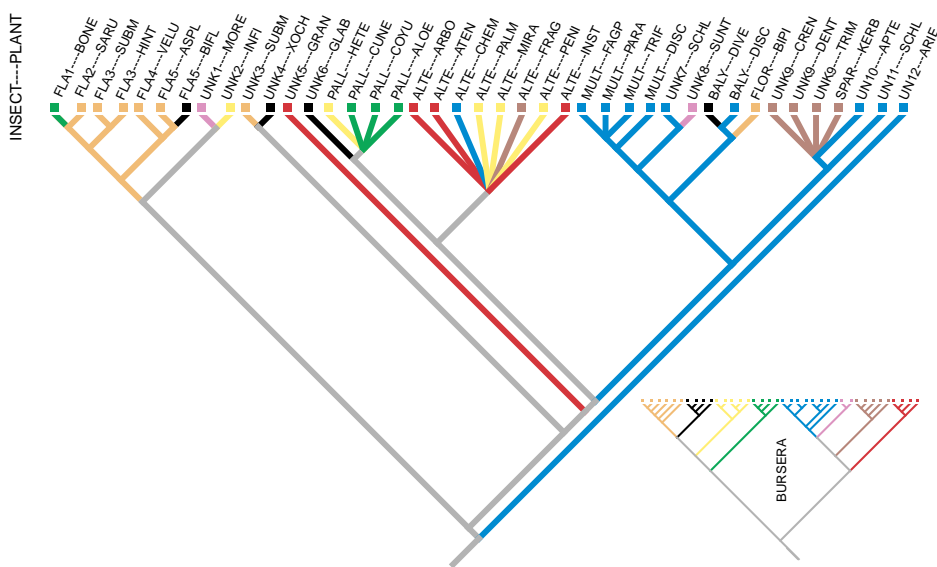


Fig. 4. Comparison of *Blepharida* and *Bursera* phylogenies. The eight major clades of *Bursera* are traced onto *Blepharida's* phylogeny. For analyses, *Bursera mirandae* and *Bursera heteresthes* were situated according to two of the four maximally parsimonious trees. *Blepharida* has shifted numerous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

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*.

REFERENCES AND NOTES

- D. G. Futuyma and M. C. Keese, in *Herbivores: Their Interactions with Secondary Plant Metabolites*, G. A. Rosenthal and M. Berenbaum, Eds. (Academic Press, San Diego, CA, 1991), vol. 2, pp. 439–473.
- P. R. Ehrlich and P. H. Raven, *Evolution* **18**, 586 (1964).
- M. Berenbaum, *ibid.* **37**, 163 (1983); D. G. Futuyma and S. S. McCafferty, *ibid.* **44**, 1885 (1990).
- B. Farrell and C. Mitter, *ibid.* **44**, 1389 (1990).
- C. Mitter, B. Farrell, D. G. Futuyma, *Trends Ecol. Evol.* **6**, 290 (1991).
- R. McVaugh and J. Rzedowski, *Kew Bull.* **18**, 317 (1965); J. Rzedowski and H. Kruse, *Taxon* **28**, 103 (1979).
- D. G. Furth and D. A. Young, *Oecologia* **74**, 496 (1988).
- D. G. Furth, *J. N.Y. Soc. Entomol.* **100**, 399 (1992).
- _____, *Spixiana* **7**, 43 (1982).
- H. A. Mooney and W. A. Emboden, *Brittonia* **20**, 44 (1968).
- J. X. Becerra and D. L. Venable, *Biotropica* **22**, 320 (1990); J. X. Becerra, *Ecology* **7**, 1991 (1994).
- J. H. Langenheim, *J. Chem. Ecol.* **20**, 1223 (1994).
- J. X. Becerra, in *Novel Aspects of the Biology of Chrysomelidae (Coleoptera)*, P. Jolivet, P. Cox, D. Petitpierre, Eds. (Kluwer Academic Press, Dordrecht, Netherlands, 1994), pp. 327–330.
- The internal transcribed spacers (ITS1 and ITS2) and the 5.8S cistron of nuclear ribosomal DNA were sequenced for 57 *Bursera* species and five outgroups following [B. G. Baldwin, *Mol. Phylog. Evol.* **1**, 3 (1992)]. The outgroups were three species of *Commiphora*, one species of *Boswellia* (Burseraceae), and one Anacardiaceae species of the genus *Spondias*. The sequences were aligned with the University of Wisconsin Genetics Computer Group software package (GCG) and Sequencher [Gene Codes Corporation Incorporated, *Sequencher 3.0 User Manual* (Ann Arbor, MI, 1995)], producing a matrix of 831 characters, of which 53.6% were potentially informative phylogenetically. Phylogenies were inferred by using parsimony analysis with PAUP 3.1.1 [D. L. Swofford, *Phylogenetic Analysis Using Parsimony (PAUP) Version 3.1.1* (Smithsonian Institution, Washington, DC, 1993)] [100 heuristic searches, random addition, Tree Bisection Reconnection (TBR) branch swapping]. A bootstrap analysis (500 bootstrap searches, 40 random additions, TBR branch swapping) was performed to estimate the relative internal support for different elements of the trees. The phylogeny of Fig. 1 includes only species on which *Blepharida* was found.
- 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*.
- 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- μ m 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.
- D. Maddison and W. Maddison, *MacClade Version 3* (Sinauer, Sunderland, MA, 1992), pp. 237–250.
- R. Page, *COMPONENT User's Manual, release 2.0* (The Natural History Museum, London, 1992).
- J. S. Farris, *Syst. Zool.* **22**, 50 (1973).
- I thank D. Furth for his help and orientation with *Blepharida* systematics and identification of specimens collected; P. Evans and J. Rodriguez for doing the gas chromatography; A. Agellon, B. Farrell, M. Hammer, D. Olmstead, M. Wojciechowski, R. Page, and the personnel from the Laboratory of Molecular Systematics and Evolution of the University of Arizona for helping with DNA sequencing techniques and statistical analyses; E. Dyreson, M. Fishbein, M. Kaplan, and S. Masta for valuable comments to earlier manuscripts; L. Venable for his help in many activities including months of field assistance; and T. Goodsell for the illustrations. Supported by NSF (INT-9505941), the Alfred P. Sloan Foundation, and the National Geographic Society.

17 July 1996; accepted 6 February 1997

Phylogenetic Analysis of Glycolytic Enzyme Expression

V. A. Pierce* and D. L. Crawford†‡

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 evi-

dence 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

Department of Organismal Biology and Anatomy, University of Chicago, 1027 East 57 Street, Chicago, IL 60637, USA.

*Present address: Department of Ecology and Evolutionary Biology, University of California at Irvine, Irvine, CA 92697–2525, USA.

†Present address: Division of Molecular Biology and Biochemistry, School of Biological Sciences, University of Missouri at Kansas City, Kansas City, MO 64110, USA. E-mail: crawd@cctr.umkc.edu

‡To whom correspondence should be addressed.