ON DEFINING "KEY INNOVATIONS" IN AN ADAPTIVE RADIATION: CYTOCHROME P450S AND PAPILIONIDAE

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Abstract.—The concept of the key innovation has been central to discussions of specialization and adaptive radiation in herbivorous insects. Rarely, however, have key innovations been defined mechanistically; typically, a key innovation is described broadly as a complex suite of traits (e.g., the ability to feed on angiosperm plants). Identifying the molecular genetic basis for individual traits that collectively characterize such key innovations can be useful in determining whether all species within a lineage share a particular key innovation or whether convergent innovations have evolved independently. Within the genus *Papilio,* the ability to feed on a furanocoumarin-containing plant has been identified as a key innovation; over 75% of the species in the genus are associated to some degree with plants containing furanocoumarins. In this group, furanocoumarin metabolism is effected by cytochrome P450 monooxygenases, heme-bound proteins known in many organisms to play a role in xenobiotic detoxification. Although furanocoumarin-metabolizing P450s in two species of *Papilio* do not share high levels of identity overall, conserved elements can be found; in particular, levels of amino acid identity in a putative substrate recognition site are very high. This conservation of amino acid identity contrasts sharply with reportedly high levels of diversification in substrate recognition sites observed in comparisons of proteins within species or between species that do not share a narrow range of host plants. Specialization on a particular group of host plants and subsequent diversification on those hosts may necessitate conservation of function in these regions, which may thus be considered key innovations in a lineage.

The evolution of a suite of traits, or key innovations, that increase the efficiency with which a resource is used has long been thought to provide entry into an adaptive zone, that is, an ecological opportunity that promotes diversification (Simpson 1953). The concept of the key innovation is an essential element of hypotheses concerning the evolution of specialization and subsequent adaptive radiation in herbivorous insects. Ehrlich and Raven (1964) postulate that biochemical novelty leads to speciation in angiosperm groups and that subsequent behavioral and physiological innovations in processing plant secondary metabolites lead to speciation in herbivorous groups associated with the biochemically distinct hosts. As they state, "If a recombination or mutation appeared in a population of insects that enabled individuals to feed on some previously protected plant group, selection could carry that line into a new adaptive zone. Here it would be free to diversify" (p. 602).

In almost all discussions of plant-herbivore interactions to date, key innovations have been broadly defined. In Ehrlich and Raven's (1964) discussion of

Am. Nat. 1996. Vol. 148, pp. S139–S155.

coevolution, association with a particular chemically distinct group of host plant taxa is considered an evolutionary innovation; Mitter et al. (1988), in fact, consider the plant-feeding habit itself to be a key innovation in the radiation of herbivorous insect taxa. Generally, adaptations are defined in broad terms behaviorally (e.g., the ability to oviposit on a particular subset of hosts; Thompson 1988) or physiologically (e.g., the ability to survive on, or even occur on, a particular group of host plants; Sperling and Feeny 1995). Considerable insight can be gained, however, by defining more precisely components of these broad traits. Such identification, for example, can contribute to the estimation of homoplasy in the acquisition of a trait within a lineage. The broad characterization of key innovations to date in the context of plant-insect interactions makes such an undertaking operationally very difficult.

As Mitter and Farrell (1992) state, "'Host use' is a compound trait, whose morphological, physiological, and behavioral components may have complex separate genetic bases" (p. 38). The use of the compound trait "host use" as the basis for designating a key innovation can be problematic inasmuch as the host records from which utilization patterns are drawn do not necessarily reflect survival and reproduction on a particular host (in fact, if a particular individual is collected from a particular host plant to authenticate a record, it perforce did not survive to reproduce). While in some instances it is clear that all members of a particular clade (or adaptive radiation) share the same key innovation, in other cases, because of the breadth of the definition, the possibility exists that related species may have evolved similar traits by convergence rather than by common ancestry.

By the same token, key innovations that are postulated to arise independently, by virtue of a particular assessment of taxonomic relationship, may on reexamination turn out to share a common origin. A case in point is the production of glucosinolates in 15 families of angiosperm plants. Before the construction of a molecular phylogeny, the biosynthesis of glucosinolates was thought to have evolved independently at least three times; molecular data indicated a much closer relationship among the disparate groups of glucosinolate-producing taxa and most likely only two independent origins (Rodman et al. 1991).

The broad nature of most key innovations, as defined in discussions of plant-insect interactions, does not necessarily make them impossible to characterize at the genetic level. Major morphological shifts in quantitative traits, for example, are now known to result in some cases from genetic changes at a single locus (Lai et al. 1995), and the possibility exists that major host shifts may result from changes at only a small number of loci (Bush 1975; White 1978). To understand the evolution of specialization, it may be useful to characterize adaptive innovations in such a way as to render them amenable to genetic analysis.

In contrast with studies of plant-insect interactions, key innovations in many pathogen-host interactions are often defined biochemically. An example of a clearly defined key innovation is a host-specific toxin produced by a pathogen that allows infection of a novel host (Kusaba and Tsuge 1994) or a detoxification enzyme that permits survival on a chemically refractory host. For example, the ability of a root-infesting cereal rust fungus, *Gaeumannomyces graminis avenae,*

to infect oats depends on the presence of a functional saponinase enzyme, avenacinase, which detoxifies the oat saponin avenacin A. When the gene encoding the enzyme is disrupted, the fungus is rendered incapable of growth on the saponin-containing oat host, although its ability to colonize other cereal hosts is unimpaired (Bowyer et al. 1995). Mortality in the absence of a functional enzyme provides compelling evidence of the importance of a single genetic trait as a key innovation in promoting fitness on a novel host.

No comparable evidence of enzyme-dependent survival on a novel or unusual host exists as yet for any plant-insect interaction. Yet specialization in individual herbivorous insects has been consistently associated with the ability to metabolize host plant toxins (Cohen et al. 1989; Nitao 1990, 1995; Berenbaum 1991*a*, 1995*a*; Lindroth 1992; Snyder et al. 1994). Molecular/genetic methods are now available to define and characterize these metabolic innovations more precisely. Particularly amenable to study are cytochrome P450 monooxygenases (P450s), xenobiotic-metabolizing heme-bound enzymes that in general convert lipophilic substances to hydrophilic and thus excretable metabolites by insertion of an activated oxygen into the substrate (Brattsten 1992). While some P450s, generally those bound to the inner mitochondrial membrane, function in hydroxylation of steroid substrates, those bound to the endoplasmic reticulum play a critical role in metabolizing and inactivating (or detoxifying) foreign substances in a wide range of organisms. Cytochrome P450s are encoded by a large gene superfamily, with over 200 described members (Nelson et al. 1993). These proteins are designated by the acronym CYP; this acronym is followed by a number that refers to a gene family (members of which share 40% or greater sequence identity). Families are divided into subfamilies (designated by a capital letter) based on sequence identity of 55% or greater; individual genes within a subfamily are indicated by a number. Gonzalez and Nebert (1990) have suggested that these enzymes have proliferated in the context of plant-herbivore interactions—that P450s with endogenous substrates, by multiple gene duplication events and subsequent mutations, acquired broader substrate specificities and, accordingly, the ability to metabolize xenobiotics. In the case of humans, it has even been suggested that reduction or elimination of plant toxins from the human diet may have led to relaxation of selection on these enzymes, which allowed for the accumulation of pseudogenes and the eventual elimination of individual obsolete genes (Kimura et al. 1989; Gonzalez and Gelboin 1992). Indeed, numerous polymorphisms documented to exist in human populations result in dramatically different rates of drug metabolism and tolerance (e.g., Skoda et al. 1988; Kimura et al. 1989; Gaedigk et al. 1991).

Cytochrome P450s are clearly involved in the metabolism of furanocoumarins, benz-2-pyrone compounds produced primarily by plants in the families Rutaceae and Apiaceae (Berenbaum 1991*b*). Cytochrome P450s responsible for the metabolic detoxification of these compounds have been identified in dogs, chickens, goats, humans, mice, rats, and species in three orders of insects (Berenbaum 1995*a*, 1995*b*, and references therein). The majority of insects associated with furanocoumarin-containing plants are oligophagous; species associated with furanocoumarin-containing umbellifers are often also associated with furanocou-

marin-containing Rutaceae or furanocoumarin-containing representatives of other families. Moreover, these insect taxa tend to be more species rich than closely related taxa associated with plants lacking furanocoumarins (Berenbaum 1983). In that this pattern conforms to the pattern described by Ehrlich and Raven (1964), a key innovation, the ability to metabolize furanocoumarins and feed on furanocoumarin-containing plants, is postulated to have led to dietary specialization and adaptive radiation (Berenbaum 1983).

The genus *Papilio* is one of the taxa in which such an adaptive radiation may have taken place. Of the 220 or more species in the genus *Papilio* (sensu lato), at least 75% have been recorded as utilizing furanocoumarin-containing plants as hosts (Berenbaum 1983). Not only are furanocoumarins characteristic of the Apiaceae and Rutaceae, the two families that constitute the bulk of host plant records for this group and that share a tremendous number of other phytochemical similarities, but they are also characteristic of a handful of hosts from phytochemically distinct and taxonomically unusual hosts. For example, *Psoralea,* the sole leguminous host plant for any species in the genus, is also one of only two leguminous genera containing furanocoumarins (Berenbaum 1983).

The Rutaceae, a family in which furanocoumarin production is widespread (Murray et al. 1982), is thought to be the ancestral host family for the genus (Hancock 1983; Scriber 1995), a relationship that is consistent with the notion that the ability to metabolize furanocoumarins is ancestral in the genus. Indeed, a survey of furanocoumarin-metabolic capabilities within the family reveals active P450-mediated metabolism only within the genus *Papilio* (Cohen et al. 1992); the inability of leptocircine or troidine swallowtails to metabolize furanocoumarins is consistent with their inability to utilize or survive on furanocoumarin-containing hosts (Berenbaum 1991*a*). Although furanocoumarin tolerance appears to be restricted to the genus *Papilio* within the tribe Papilionini, whether this tolerance arose early in the evolution of the group or multiple times by convergence has not been established.

The large genus *Papilio* has traditionally been divided into five sections (Munroe 1961); species that feed regularly on furanocoumarin-containing plants are found in Sections II, III, and IV. Within Section II is the black swallowtail *Papilio polyxenes,* a species that feeds primarily on furanocoumarin-containing umbelliferous or rutaceous hosts (Berenbaum 1981). The black swallowtail is capable of high levels of P450-mediated metabolism of furanocoumarins; moreover, levels of metabolism are up to eightfold inducible by dietary exposure to these compounds (Cohen et al. 1989). The isozyme responsible for much of this metabolism, CYP6B1, exists as at least two allelic variants in *P. polyxenes* (Cohen et al. 1992). Expression of this P450 in lepidopteran cells using a baculovirus expression system has revealed that the isozymes encoded by these cDNAs are capable of metabolizing linear furanocoumarins in a manner consistent with that displayed by intact insects (Ma et al. 1994). A related cDNA, *CYP6B3,* has been cloned and characterized from caterpillars exposed to dietary angelicin, an angular furanocoumarin, and is thought to represent a second P450, possibly more effective at metabolizing angular furanocoumarins. In contrast to CYP6B1, it is induced in vivo by a wide range of furanocoumarins, including both linear and

angular forms (Hung et al. 1995*a*, 1995*b*). Determining the substrate specificity of CYP6B3 awaits expression in a heterologous system.

Even these preliminary findings illustrate the potential informational value of categorizing constituent elements of key innovations. Sperling and Feeny (1995), for example, argue that angular furanocoumarin feeding is ancestral in the *machaon* complex of the genus *Papilio,* based on host records; ostensibly, then, a common mechanism for metabolizing angular furanocoumarins is shared by all members of the group (except for those in which it has been lost secondarily). Yet host records on angular furanocoumarin-containing host plants for all of these species may be misleading in that they provide little information on the relative performance of these species on angular furanocoumarin-containing plants, the relative frequency with which angular furanocoumarin-containing plants are utilized by these species, or the relative metabolic capabilities of the dozen or so species in this complex against angular furanocoumarins. Nor do these host records provide information on the relative content of angular furanocoumarins in the plant parts consumed (even within a single species, angular furanocoumarins are not necessarily produced by all individuals or by all parts within an individual plant; Berenbaum 1981). In the black swallowtail, CYP6B1 has a limited ability to metabolize angular furanocoumarins (Ma et al. 1994; Hung et al. 1995*a*); other P450s (possibly CYP6B3) are also likely involved in angular furanocoumarin metabolism. Among the other members of the *machaon* complex (the group within Section II to which *P. polyxenes* belongs) is *Papilio brevicauda,* the short-tailed swallowtail, which tends to feed more extensively on angular furanocoumarin-containing plants than does its close relative *P. polyxenes* (Berenbaum and Feeny 1981). The possibility exists that the "key innovation" that allows *P. brevicauda* to colonize angular furanocoumarin-containing umbelliferous hosts (Berenbaum and Feeny 1981) may involve a P450 locus different from the one(s) on which *P. polyxenes* relies to detoxify furanocoumarins in its broader range of hosts. Some evidence exists that the P450s in these two species, although similar, are not necessarily orthologous. Reactivities against particular furanocoumarins are not identical, nor, in Northern analysis, do mRNAs produce identical signals at high stringencies when screened with the black swallowtail *CYP6B1* (Cohen et al. 1992).

More dramatic divergence among furanocoumarin-associated P450s exists within the genus as a whole. While *CYP6B1* cDNA is cross-reactive and detectable at high stringencies with mRNAs from furanocoumarin-induced mRNAs from *P. brevicauda,* a close relative of *P. polyxenes* in the *machaon* complex, *CYP6B1* cDNA is cross-reactive only at low stringencies with mRNAs from xanthotoxin-induced larvae of *Papilio cresphontes,* a more distantly related Section IV Rutaceae specialist that normally encounters furanocoumarins in its hosts, and *Papilio glaucus,* a polyphagous species in Section III that rarely if ever encounters furanocoumarins in its hosts (Cohen et al. 1992).

A cDNA encoding the furanocoumarin-metabolizing P450 from *P. glaucus* has been cloned and characterized in a baculovirus expression system (C.-F. Hung, M. R. Berenbaum, and M. A. Schuler, unpublished manuscript). Although the CYP6B4 protein shares only 63% amino acid identity with CYP6B1, both proteins metabolize significant levels of linear furanocoumarins. Whereas the rank order

of linear furanocoumarin metabolism differs for the two proteins, both proteins metabolize linear furanocoumarins more rapidly than angular furanocoumarins (C.-F. Hung, M. R. Berenbaum, and M. A. Schuler, unpublished manuscript). Despite these overall similarities in metabolic activity, these enzymes appear to be distantly related to one another. Indeed, *P. glaucus* itself may be only distantly related to *P. polyxenes; P. glaucus* and other Section III species (Munroe 1961) have occasionally been placed in the separate genus *Pterourus* (Tyler et al. 1994).

This overall low level of similarity does not rule out the possibility that these two enzymes are related and constitute a shared "key innovation." Similarity of function may be due to conservation in particular regions of the enzyme, such as those regions responsible for recognition and binding of substrates; these regions may well be subject to stabilizing selection within a lineage. Unfortunately, the catalytic site for CYP6B1 has not yet been defined. The X-ray three-dimensional structure is known for a handful of bacterial P450s (Hasemann et al. 1995); of these, *Pseudomonas putida* $P450_{cam}$ (CYP101) has been extensively used as a structural model for eukaryotic P450s in the absence of any eukaryotic crystallographic structures. Based on sequence alignment with $P450_{cam}$, Gotoh (1992) was able to identify six putative substrate recognition sites (SRSs) in CYP2 proteins (but see Hasemann et al. 1995 for a discussion of the limitations of this approach). A considerable body of work, in which site-directed mutagenesis is used to substitute amino acids in these putative substrate recognition sites, has confirmed their importance in determining substrate specificities (e.g., Lindberg and Negishi 1989; Johnson 1992; Mayazumi et al. 1993; Straub et al. 1994; He et al. 1995).

Aligning these sequences and identifying substrate recognition sites allowed Gotoh (1992) to examine intraspecific variation at these SRS regions to determine whether they are subject to diversifying or stabilizing selection using the method of Hughes and Nei (1988) for learning whether polymorphic enzymes are under positive selection by examining nucleotide substitution patterns. In this analysis, if critical regions of the enzyme are under positive selection, then the rate of nonsynonymous, or amino acid–changing, substitutions in the gene should be higher than the rate of synonymous substitutions (Nei and Gojobori 1986). Regions where synonymous substitutions equal nonsynonymous substitutions are presumably experiencing neutral evolution, and regions where synonymous substitutions exceed nonsynonymous substitutions are undergoing purifying (stabilizing) selection. At least a dozen cases are documented (Lee et al. 1995) for intralocus comparisons of allelic variation. Positive selection is consistently demonstrated in proteins involved in extracellular recognition processes, including P450s (Gotoh 1992). Intralocus polymorphism may be expected to be particularly high in xenobiotic-metabolizing P450s, considering the almost limitless number of potential environmental toxicants that may act as selective agents on these proteins.

To examine the idea that particular substrate recognition sites may constitute key innovations in detoxification enzymes within a lineage, we performed a series of analyses. Initially, we performed phylogenetic analyses to determine the degree to which these P450s are related. Identifying relationships among enzymes

TABLE 1

SUMMARY OF GENES AND THEIR SOURCES USED IN ANALYSIS

Gene	Scientific Name	Common Name	Furanocoumarin Metabolism Confirmed
Cyp2a-5	*Mus musculus*	Mouse	Yes
CYP4C1	*Blaberus discoidalis*	Tropical cockroach	No
CYP6B1	*Papilio polyxenes*	Black swallowtail	Yes
CYP6B2	*Helicoverpa armigera*	Cotton bollworm	No
CYP6B3	*P. polyxenes*	Black swallowtail	Yes
CYP6B4	*Papilio glaucus*	Tiger swallowtail	Yes
CYP6A1	*Musca domestica*	House fly	No
CYP6A2	*Drosophila melanogaster*	Fruit fly	No
CYP6A3	*M. domestica*	House fly	No
CYP6A4	*M. domestica*	House fly	No
CYP6A5	*M. domestica*	House fly	No
CYP6C1	*M. domestica*	House fly	No
CYP6D1	*M. domestica*	House fly	No
CYP9A1	*Heliothis virescens*	Tobacco budworm	No

(e.g., such as orthology, sharing by two species prior to evolutionary divergence) has proved exceedingly difficult for P450s because of the diversity of substrates metabolized even by extremely similar enzymes (Gonzalez and Gelboin 1992), similar function being one potential indicator of orthology (Fitch 1970). Standard phylogenetic analysis may provide a method for determining the likelihood of shared ancestry for particular sets of enzymes. For P450s that appear to be orthologous, we then undertook an analysis of nucleotide and amino acid sequence similarity both within and between substrate recognition sites, to determine whether these sites have experienced stabilizing selection, as might be expected for species that have diversified on a phytochemically similar set of hosts, or diversifying selection, as would occur if unrelated xenobiotics independently exerted selection pressure.

METHODS

Sequence Alignment

To determine patterns of relationship among members of the CYP6B subfamily, we obtained protein sequence data for CYP6B1v1, CYP6B1v2, CYP6B3 (*Papilio polyxenes:* Cohen et al. 1992; Hung et al. 1995*a*), CYP6B2 (*Helicoverpa armigera:* Wang and Hobbs 1995), CYP6B4 (*Papilio glaucus:* C.-F. Hung, M. R. Berenbaum, and M. A. Schuler, unpublished manuscript), CYP6A2 (*Drosophila melanogaster:* Waters et al. 1992), CYP6A1, CYP6A3, CYP6A4, CYP6A5 (*Musca domestica:* Cohen and Feyereisen 1995), CYP6C1, CYP6D1 (*M. domestica:* Tomita and Scott 1995), CYP4C1 (*Blaberus discoidalis:* Bradfield et al. 1991), CYP9A1 (*Heliothis virescens:* Rose et al. 1995), and CYP2a5 (*Mus musculus:* Lindberg and Negishi 1989) from GenBank and aligned with reference to the mammalian CYP2a5, one of the sequences with SRS regions demarcated by Gotoh (1992) that, like CYP6B1 and CYP6B4, possesses furanocoumarin-metabolizing ability (Maenpaa et al. 1994) (table 1). Sequences averaged 500–600 bases in length;

these sequences were aligned with the use of the pileup command in the program GCG (Devereux et al. 1984). Gap weight was assigned at 3.00 and gap length weight at 0.100; these gap weight assignments are the default option but proved sufficient for our purposes in that the number of gaps resulting after alignment was not excessive.

Maximum-Parsimony Analysis

Maximum-parsimony analysis (PAUP version 3.1.1; Swofford 1993) was used to estimate relationships among putative furanocoumarin-metabolizing enzymes and other insect P450s. No assumptions about polarities were made in this analysis. The gene Cyp2a5, from mouse, was designated as an outgroup for rooted trees because it is known to possess both coumarin- and furanocoumarin-metabolizing abilities. To provide greater depth to the analysis, insecticide resistance–associated P450s from *Musca domestica* and *Drosophila melanogaster* were included in the phylogenetic analyses. Most parsimonious trees were also estimated using CYP4C1 (from the cockroach *Blaberus discoidalis*) and CYP9A1 (from the tobacco budworm *Heliothis virescens*) as an outgroup and with all three outgroups combined paraphyletically in an effort to reduce the taxonomic distance between in-group and outgroup, which can produce inconsistencies in maximum-parsimony analyses. Relationships may be obscured if the branch length of the outgroup is substantially greater than branches in the in-group (Felsenstein 1978). One way to ameliorate this problem is to increase the number of taxa in the outgroup (Swofford and Olsen 1990; Smith 1994).

After unweighted maximum-parsimony analyses were conducted, trees were produced in which amino acid sequences in the substrate recognition sites, as designated by Gotoh (1992), were more heavily weighted than amino acid sequences outside these regions. The rationale for the weighting procedure is that similarities in the substrate recognition sites are most likely to reflect similarity of function. One perennial problem in analyzing members of a multigene family (such as the P450s) is that the identification of orthologous proteins and similar function may be a potential indicator of orthology (Fitch 1970). Weighting factors ranging from three to 20 were used in the analysis. All analyses were performed using heuristic searches with tree bisection-reconnection branch swapping. Character evolution was optimized using the accelerated transformation option. A bootstrap analysis, with 200 replicates, was also conducted to test the robustness of the phylogenetic analysis (Swofford 1993).

Rates of Synonymous versus Nonsynonymous Substitution in Substrate Recognition Sites

The method of Hughes and Nei (1988) was used to compare DS/DN (synonymous substitutions/nonsynonymous substitutions) for different putative substrate recognition sites encoded in *CYP6B* genes. In interpreting these analyses, previous authors have suggested that an excess of nonsynonymous substitutions in substrate (or antigen) recognition sites is evidence of diversifying (or positive Darwinian) selection, resulting from coevolutionary interactions between organisms. This pattern has been documented in major histocompatibility complex

antigen recognition sites (Hughes and Nei 1988; Hughes 1992), in polydnavirus/wasp interaction-mediating proteins (Summers and Dib-Hajj 1995) and in sperm acrosomal proteins (Lee et al. 1995; Swanson and Vacquier 1995). In contrast, an excess of synonymous substitutions suggests the presence of stabilizing selection. Such a pattern was documented for regions of CYP2 proteins outside substrate recognition sites and is thought to encode structural features of the protein (e.g., beta sheets and helices) necessary for function (Gotoh 1992). Nucleotide sequence data were obtained for CYP6B1v1, CYP6B1v2, CYP6B3, CYP6B2, and CYP6B4 from GenBank and aligned with the use of the pileup command in the program GCG (Devereux et al. 1984).

In addition to analyses conducted on nucleotide base sequence similarity, χ^2 analysis was used to compare the percentage amino acid identity within and outside substrate recognition sites. The number of state changes for each character was also determined in an effort to judge different levels of amino acid conservation in different regions of the sequences.

RESULTS

Maximum-Parsimony Analysis

The unweighted maximum-parsimony analysis with 2a5, 4C1, and 9A1 as the outgroup resulted in a single maximum-parsimony tree 2,758 steps long (confidence interval [CI] = 0.788). This tree confirms the close relationship between CYP6B1 and CYP6B3, both isolated from *Papilio polyxenes,* the black swallowtail. We found that CYP6B4, a furanocoumarin-metabolizing P450 isolated from *Papilio glaucus,* grouped with the other swallowtail sequences, which suggests that the *Papilio* CYP6B P450s resulted from a duplication of an ancestral P450 not shared by noctuids (e.g., CYP6B2) (fig. 1). However, this interpretation is necessarily limited by the availability of lepidopteran sequences to use in analyses; the incorporation of P450 sequences from a large number of *Papilio* species could conceivably alter the perceived close relationships. Weighting sequences within the substrate recognition sites did not appreciably affect the shape of the cladogram, nor did rooting the tree with CYP4C1, an insect P450 not known to play any role in xenobiotic metabolism (fig. 2). The only conspicuous effect of weighting SRS1 in this analysis was to change the position of CYP6D1 within the tree; although derived from the house fly, it groups with other dipteran P450s only with weighting factors of five or higher.

Rates of Synonymous versus Nonsynonymous Substitution in Substrate Recognition Sites

Saturation at synonymous sites prevented a statistical analysis of codon usage in many of the comparisons of *CYP6B* cDNA. We chose to focus on SRS1 because such a striking level of similarity among swallowtail sequences was revealed in this region. Although there were on average 46.5 possible sites for nonsynonymous substitutions in SRS1, no nonsynonymous substitutions were found in comparisons of *CYP6B1* and *CYP6B3*. The ratio of synonymous to non-

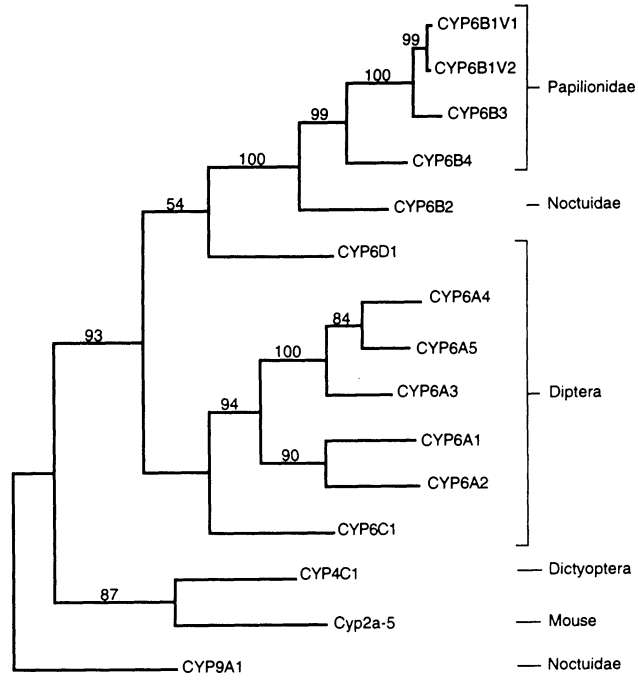

FIG. 1.—Cladogram depicting relationships among insect P450 proteins (Cyp2a5 from mouse, CYP4C1 from *Blaberus discoidalis,* and CYP9A1 from *Heliothis virescens* as paraphyletic outgroup). Numbers on branches are the percentage of trees showing that particular grouping in bootstrap analysis; tree length = 2,758 steps; CI = 0.788.

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          101                                                  150
CYP6B1V1  KDFESFADRG V--EFSLDGL GANIFHADGD RWRSLRNRFT PLFTSGKLKS
CYP6B1V2  KDFESFADRG V--EFSLDGL GANIFHADGD RWRSLRNRFT PLFTSGKLKS
  CYP6B3  KDFEPFADRG V--EFSLDGL GANIFHADGD RWRSLRNRFT PLFTSGKLKT
  CYP6B4  KDFDLFNDRG V--EFSEEGL GLNIFHADGD RWRVLRQCFT PLFTSGKLKN
  CYP6B2  KDFEVFSDRG L--EFSKEGL GQNLFHADGD TWRTLRNRFT PIFTSGKLKN
  CYP6A4  KDFANFHDRG GLSNVEDDPL TGHLVSLEGE QWRAMRTKLT PVFTSARMKY
  CYP6A5  KDFANFHDRG VFNNVEDDPL TGHLVALEGE QWRAMRTKLS PVFTSARMKY
  CYP6A3  KDFAYFQNRG GFHNVEDDPL TGHLSNLEGE QWRALRAKLT PVFTSARMKY
  CYP6A1  KDFSNFANRG LYYNEKDDPL TGHLVMVEGE KWRSLRTKLS PTFTAGKMKY
  CYP6A2  KDFSNFADRG QFHNGRDDPL TQHLFNLDGK KWKDMRQRLT PTFTSGKMKF
  CYP6C1  TEFEVFPDRG FFVNYKSDPL SRNMARLHGE MWRKIRTKIT PTFTAAPMRQ
  CYP6D1  TDFNSFHDRG LYVDEKNDPM SANLFVMEGQ SWRTLRMKLA PSFSSGKLKG
 Cyp2a-5  DQAEEFSGRG EQATFDWLFK GYGVVFSSGE RAKQLRR--- --FSIATLRD
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FIG. 2.—Putative substrate recognition site 1 in insect P450s, in comparison with Cyp2a-5 (Gotoh 1992).

TABLE 2

PERCENTAGE AMINO ACID IDENTITY IN SWALLOWTAIL P450S

Sequence Region	6B1 versus 6B3	6B1 versus 6B4	6B3 versus 6B4
Entire sequence	87.75	63.25	61.85
SRS regions only	89.39	62.32	63.08
Sequence minus SRSs	87.08	61.74	59.95
SRS1 only	100.00	85.00	85.00
Sequence minus SRS1	87.23	62.34	60.88

synonymous substitutions in SRS1 in a comparison of *CYP6B1* and *CYP6B4* was 4.73, and of *CYP6B3* and *CYP6B4,* 5.28; both values are well in excess of those to be expected in cases of neutral or positive selection. In cases in which nonsynonymous substitutions are lacking, amino acid identity can be used as an alternative means of determining whether elements of a protein associated with a common function are conserved within a lineage. In SRS1 (fig. 2; table 2), amino acid identity between CYP6B1, in *P. polyxenes,* and CYP6B4, in *P. glaucus,* was 85%, significantly higher than the overall percentage identity (63%) or the percentage identity in the sequence exclusive of SRS1 (χ^2, $P < .01$).

Confirmation of the highly conserved nature of SRS1 relative to the rest of the amino acid sequence is evidenced also by the low level of character-state changes across SRS1 and extending beyond (roughly from residue 70 to 145; fig. 3).

DISCUSSION

Contrary to prior reports on other proteins involved in interspecific interactions, our study suggests that P450s involved in furanocoumarin metabolism in papilionid larvae appear to display high levels of amino acid identity in at least one putative substrate recognition site. Within a lineage, genic-encoded substrate recognition sites of P450s involved in mediating trophic interactions do not necessarily possess high rates of nonsynonymous substitutions (Gotoh 1992). In addition to selection pressure for diversification of function, then, P450s may also be subject to stabilizing selection. It is likely that SRS1 is involved in substrate binding; in fact, site-directed mutagenesis of F116 to Y in this region reduces xanthotoxin metabolic activity by at least 90% (M. Pookanjanatavip, unpublished manuscript). This position corresponds to V117 in CYP2a5, which has been shown by site-directed mutagenesis to be involved in coumarin metabolism in the mouse (Lindberg and Negishi 1989).

Other conserved elements may exist within this gene family. Despite the fact that *Papilio glaucus* rarely encounters furanocoumarins, many aspects of its furanocoumarin-metabolic capabilities resemble those of *Papilio polyxenes*. At constitutive levels, metabolism of linear furanocoumarins exceeds that of angular furanocoumarins, as is the case for black swallowtails; also, furanocoumarin metabolism in both species is substrate inducible (C.-F. Hung, M. R. Berenbaum, and M. A. Schuler, unpublished manuscript). Similarities in inducibility suggest

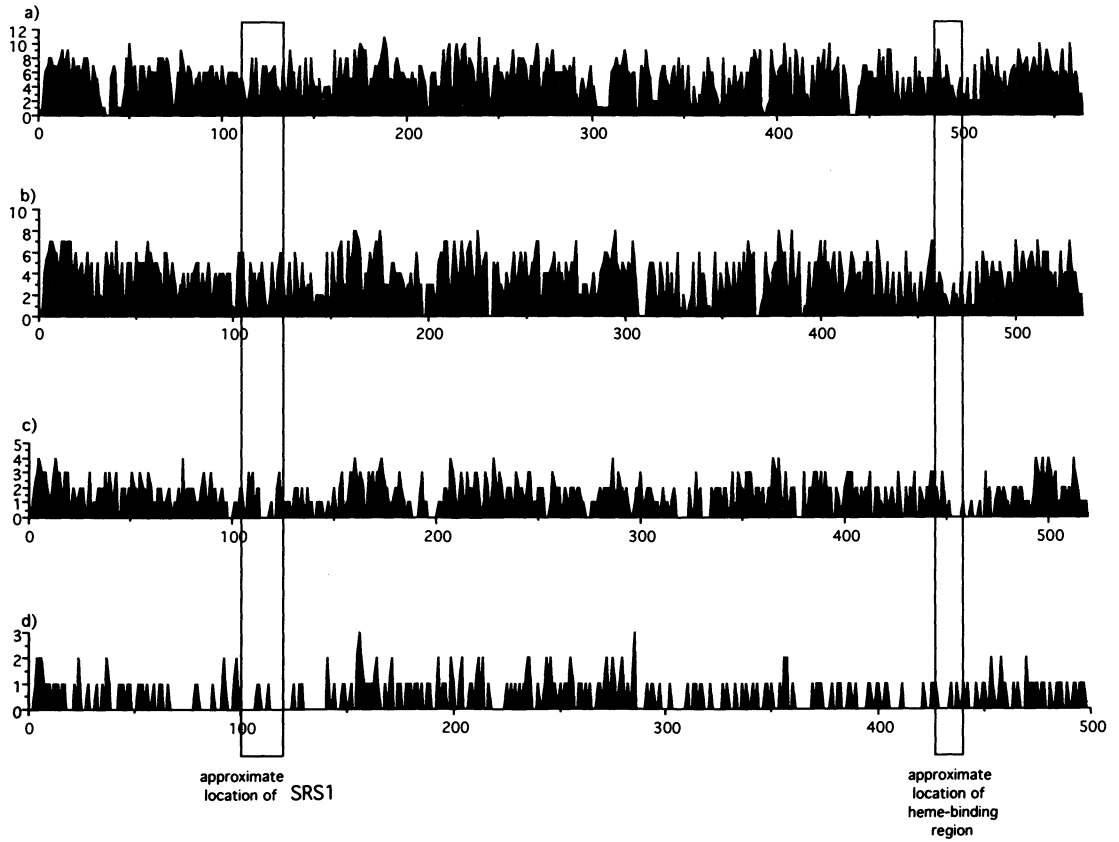

FIG. 3.—Graphic representation of character state changes in P450 amino acid sequences: *a*, all 15 sequences; *b*, all sequences in the CYP6 family; *c*, all sequences in the CYP6A subfamily; *d*, all swallowtail sequences in the CYP6B subfamily (i.e., excluding CYP6B2).

that certain regulatory elements and structural elements may be conserved within a lineage. The black swallowtail possesses a positive xanthotoxin-responsive element located between −136 and −119 that is necessary for both basal and xanthotoxin-inducible transcription of the *CYP6B1v3* gene (Prapaipong et al. 1994*a*, 1994*b*; Prapaipong 1995; H. Prapaipong, M. R. Berenbaum, and M. A. Schuler, unpublished manuscript). An element of the promoter sequence of *CYP6B4* shows substantial identity with the −136 to −119 region of *CYP6B1* (R. Holzmacher, C.-F. Hung, and M. A. Schuler, unpublished manuscript), which raises the possibility that this sequence in *CYP6B4* also functions as a xanthotoxin-responsive element.

Despite striking similarities in both structural and regulatory elements of *CYP6B* genes in swallowtail caterpillars, it cannot be credibly argued that furanocoumarin-metabolic activities alone were responsible for diversification within the genus *Papilio*. Single genes, or even a single family of genes, almost certainly cannot be solely responsible for host shifts and subsequent specialization. Many examples exist, even for papilionids, in which host finding and larval survival within a species, both necessary for a host shift, are genetically unlinked (Thompson 1988). But efforts to elucidate innovations at the genetic level and then to examine them throughout a lineage can provide great insights into mechanisms that allow insects to maintain specialized diets. For example, swallowtail larvae may well rely on cytochrome P450s to detoxify furanocoumarins, but these compounds appear to be largely irrelevant to the adult swallowtails. Adults do not seem to display any behavioral response to these compounds (P. Feeny, personal communication; M. Berenbaum, personal observation), nor do they metabolize them to any measurable extent (M. Berenbaum and A. Zangerl, personal observation). Rather, oviposition cues in swallowtail species associated with furanocoumarin-containing hosts in the Rutaceae and Umbelliferae are generally an admixture of cyclitols, flavonoids, bases, and acids (Feeny 1995; Nishida 1995). Although different compounds have been identified as being important kairomones in different species, in many species within the genus *Papilio* the same classes of compounds are involved. Thus, while any individual compound is largely uninformative in understanding the phylogeny, the receptor protein(s) responsible for binding to these compounds may display conserved features throughout the lineage. Thus, it may be the receptor that is the "key innovation" underlying the behavior. Identifying the molecular/genetic basis for the behavioral and physiological attributes that distinguish oligophagous species from those with a less restricted diet can contribute mightily to clarifying the mechanisms that underlie host shifts and the processes that lead to diversification within a lineage.

ACKNOWLEDGMENTS

We thank A. Hughes for kindly sharing software and C.-F. Hung for sharing unpublished data. In addition, we thank S. Berlocher, F. Soto, G. Olsen, and H. Robertson for advice and guidance on methods of phylogenetic analysis—H. Robertson in particular for his intellectual generosity in this regard. This work

was supported in part by U.S. Department of Agriculture National Research Initiatives grant AG92-37302-7639.

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Associate Editor: Mark D. Rausher