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Evolution of Regulatory Genes and Patterns: Relationships to Evolutionary Rates and to Metabolic Functions

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Abstract. In an effort to understand the forces shaping evolution of regulatory genes and patterns, we have compared data on interspecific differences in enzyme expression patterns among the rapidly evolving Hawaiian picture-winged *Drosophila* to similar data on the more conservative *virilis* species group. Divergence of regulatory patterns is significantly more common in the former group, but cause and effect are difficult to discern. Random fixation of regulatory variants in small populations and/or during speciation may be somewhat more likely than divergence driven by selection. Within the picture-winged group, we also have compared enzymes that fulfill different metabolic roles. There are highly significant differences between individual enzymes, but no obvious correlations to functional categories.

Key words: Evolution — Gene regulation — *Drosophila* — Adaptation — Enzymes

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Introduction

Changes in gene regulation probably are important in adaptive evolution, and the limited relevant data suggest that interspecific regulatory differences are relatively common (Wilson 1976; MacIntyre 1982; Dickinson 1991). However, there is little evidence that most regulatory divergence has been driven by selection (Dickinson 1988, 1991). Unfortunately, the few cases for which adaptive scenarios have been proposed are unlikely to be representative of the technical and conceptual problems to be confronted if we seek generalizations concerning the frequency and importance of adaptive regulatory shifts. For example, elevated stomach lysozyme in ruminants apparently supports the dietary specialization of that group (Dobson et al. 1984), and high alcohol dehydrogenase activity in adult *Drosophila melanogaster* may permit exploitation of fermented foods (Dickinson et al. 1984). In those cases, plausible hypotheses were based on relevant information about the physiological role of the gene product and the ecology of the organism, and the relationship between phenotype and function is relatively direct. For most genes whose regulatory patterns have been compared across species, no comparable background is available, and many adaptive roles may be subtle and indirect. Moreover, there is fundamental asymmetry in the ability to test alternatives. Specific adaptive explanations can be tested

and, potentially, rejected. However, the hypothesis that there is *some* adaptive function can never rigorously be rejected: It always is possible that the relevant context has not been identified. In other words, neutrality is impossible to prove in any individual case.

In seeking alternative approaches, we might profit from the extensive work on structural genes. Lewontin (1985) has analyzed the failure of massive efforts devoted to structural polymorphisms to produce a consensus on the relative importance of selection and drift, and he argues for DNA sequence data as an alternative more likely to separate those factors. One approach exploits comparisons between well-defined sites that are subject to different forces: introns vs exons, synonymous substitutions vs replacement substitutions, etc. Unfortunately, our present understanding of regulatory mechanisms provides no equally sharp distinctions at the level of DNA sequence; perhaps none exist. Comparisons between intraspecific polymorphism and interspecific substitutions provide relevant information without necessary reference to function (Kreitman 1991), but this approach may lack the resolution to separate *cis*-acting regulatory sites from linked coding sequences. Furthermore, regulation typically involves multiple transcription factors encoded at diverse loci, so any approach based on sequences is likely to ignore a large part of the system relevant to any particular gene.

If not sequences, what sorts of comparisons might help sort out the forces and constraints governing regulatory evolution? We propose two: (1) Between taxa that have experienced different evolutionary forces and (2) between genes whose products fulfill different roles. Previously, we analyzed regulatory divergence for a set of five enzymes among species of the picture-winged group of Hawaiian *Drosophila* (Dickinson 1980). We now add data on the same set of enzymes in the *Drosophila virilis* species group, which has far fewer species and considerably less morphological and ecological diversity (Carson and Yoon 1982; Throckmorton 1982). We find that the slower evolution of the *virilis* group correlates with reduced regulatory divergence. We also report data on six additional enzymes in Hawaiian species. These represent a class of metabolic functions possibly subject to greater (or at least different) selective constraints than are the enzymes included in the earlier study. There are dramatic differences between individual enzymes, but the two sets are not significantly different, nor is any other relationship to function evident to us.

Materials and Methods

Samples and Assays. Stocks of the 12 described species in the *virilis* group were obtained from the *Drosophila* Species Stock Center at Bowling Green State University. The tissues and enzymes examined were the same as in our earlier study of picture-winged *Drosophila* (Dickinson 1980). Those enzymes were alcohol dehydrogenase, octanol dehydrogenase, xanthine dehydrogenase, aldehyde oxidase 1, and aldehyde oxidase 2. The enzyme assays also were as previously reported except that they followed electrophoresis on native 10–15% polyacrylamide gels in the Pharmacia PhastSystem.

The picture-winged species (with stock numbers) for which data on a new set of enzymes are reported are *D. affinidisjuncta* (S36G1), *D. crucigera* (U72Y8), *D. grimshawii* (G1), *D. heteroneura* (T94B18), *D. silvarentis* (U87G6), *D. silvestris* (T94B7), *D. hawaiiensis* (J14B8), and *D. hirtipalpis* (J10B5). The stages and tissues chosen for analysis and the methods of sample preparation were as previously reported (Dickinson 1980).

These enzymes also were assayed following electrophoresis. This permitted resolution of distinct allozymes so that we could be sure that we assayed products of the same structural genes in the various tissues. When multiple allozymes were detected, only one major band was scored. Relative electrophoretic mobility also allowed identification of homologous gene products in different species (Dickinson 1980), and we confirmed that our identifications were the most conservative possible with respect to patterns of regulation.

Tissue extracts were electrophoresed on native 10–15% gradient polyacrylamide gels in the Pharmacia PhastSystem, except hexokinase was analyzed on 8–25% gradients. Sample size was 1 μl, and each set of extracts (from one fly) was used for up to four assays. Gels were incubated 1–3.5 h (standardized for each enzyme) at 37°C in freshly prepared stain. The new enzymes assayed (and the sources of staining procedures) were as follows: aldolase and malic enzyme (Ayala et al. 1972); D-3-hydroxybutyrate dehydrogenase (Ayala et al. 1974); alpha-glycerophosphate dehydrogenase and malate dehydrogenase (Shaw and Koen 1968); and hexokinase (Prakash 1973).

All enzyme activities were estimated by visual comparison to standard twofold dilution series similarly electrophoresed and stained (Dickinson 1980). In this system, 0 indicates no detectable activity, 1 is the last dilution at which activity could be detected, and each successive integer represents twofold-greater activity.

Data Analysis. We use the following definitions and procedures to make comparisons. A trait is the level of one enzyme in one tissue. The consensus value for a trait in a species is that which includes the largest number of determinations within ± 1 step in the scale based on twofold dilutions (above). At least four individuals were scored initially. In most cases, all fell within a consensus range. If necessary, additional individuals were analyzed to find a consensus. We similarly established a group consensus for each trait in each species group. This serves in lieu of a hypothesis concerning the ancestral state and is conservative in that it leads to a minimum estimate of divergent cases. The group consensus is the value that includes the largest number of species within ± 1 step on our scale. This procedure yields the following parameters: (1) for each trait, the number of species included within the consensus (conserved cases) and the number outside that range (divergent cases); (2) for each species, the number of conserved and divergent (non consensus) traits; (3) for a group of species, the number of variable and of invariant traits. We also determined the number of species falling further from the consensus, directions of divergence, and the range of values for each trait.

Statistical Treatment. The conserved and divergent cases for any two data sets can be represented as a 2 × 2 contingency table, so differences in frequency of these classes are conve-

Table 1. Summary of conserved and divergent regulatory traits in the picture-winged species group—enzymes likely to have exogenous substrates: the enzymes assayed were alcohol dehydrogenase (ADH), octanol dehydrogenase (ODH), xanthine dehydrogenase (XDH), and two aldehyde oxidases (AO1 and AO2)

	ADH	ODH	XDH	AO1	AO2	Total
Conserved cases	262	300	302	267	284	1415
Divergent cases	45	7	5	40	23	120
≥3 steps diverged	25	1	1	17	8	52
≥4 steps diverged	15	0	0	12	2	29
Positive divergence	15	5	5	34	20	79
Negative divergence	30	2	0	6	3	41

niently evaluated by the chi-square test. We also calculated the G statistic, which is recommended for 2 × 2 tables in which two of the marginal totals are set by the experimental design (Sokal and Rohlf 1981). The results were essentially identical, so only chi-square values are reported. Since our parameters cannot be assumed to have normal distributions we used the nonparametric Mann-Whitney U test (Sokal and Rohlf 1981) to evaluate differences in the number of divergent traits per species and in the distributions of ranges.

Results

Species Group Comparisons

New data are tabulated in the appendix and summarized, together with earlier data (Dickinson 1980) in Tables 1 and 2. The frequency of divergent cases within the picture-winged group (7.8%) is greater than in the *virilis* group (5.0%), and the difference is significant ($\chi^2 = 6.03, P < 0.025$). The trend is more pronounced for more extreme divergence: 3.39% vs 1.46% at three steps (eightfold) from the consensus and 1.89% vs 0.80% at four steps. The distributions of divergent traits across species (Fig. 1) provide an alternative way to view these data. The difference is significant ($P < 0.05$) by the Mann-Whitney U test (based on rank order). The frequency of variable traits (at least one divergent species in the group) also is higher among the picture-winged flies (43% to 32%), but this is not statistically significant ($\chi^2 = 1.6, P > 0.1$). Furthermore, the difference in number of species examined in the two groups would be expected to bias this comparison. Finally, Fig. 2 compares the ranges (twofold steps from lowest species to highest) for the 65 traits examined in the two species groups. The distribution is somewhat flatter for the picture-winged group and has a larger tail to the high end, but the difference is not significant (Mann-Whitney U test, $P > 0.5$).

Divergent cases are not uniformly distributed across enzymes. The difference between groups is accounted for almost entirely by alcohol dehydrogenase and aldehyde oxidase 2. For the former,

Table 2. Summary of conserved and divergent regulatory traits in the *virilis* species group: the enzymes assayed were the same as reported in Table 1

	ADH	ODH	XDH	AO1	AO2	Total
Conserved cases	148	154	151	121	142	716
Divergent cases	8	2	5	22	1	38
≥3 steps diverged	1	0	0	10	0	11
≥4 steps diverged	0	0	0	6	0	6
Positive divergence	2	1	3	16	0	22
Negative divergence	6	1	2	6	1	16

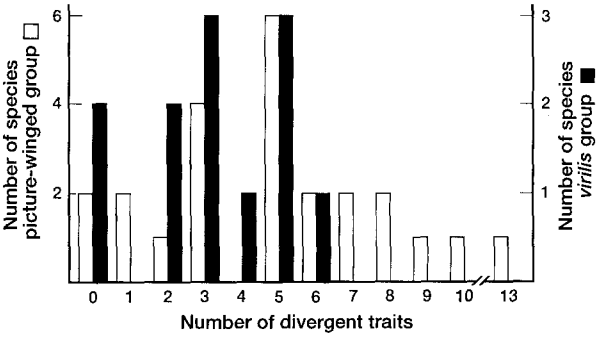

Fig. 1. The distribution of divergent regulatory traits among species. The number of species in each class is displayed as a function of the number traits in each species found to differ significantly from a group consensus phenotype. Species of the picture-winged group (*open bars*) are compared to the *virilis* group (*solid bars*). The *vertical scales* for the two groups are adjusted to compensate for the different numbers of species studied.

14.7% of the cases are divergent in the picture-winged flies, but only 5.1% are in the virilis group ($\chi^2 = 9.5, P < 0.005$). For aldehyde oxidase 2, the figures are 7.5% and 0.70%, respectively ($\chi^2 = 8.9, P < 0.005$). Xanthine dehydrogenase and octanol dehydrogenase are conservative in both groups, and aldehyde oxidase 1 has a relatively high frequency of divergence in both (13.0% and 15.4%).

Enzyme Comparisons

With respect to regulatory diversity, the new set of enzymes for which data on the Hawaiian flies are added (Appendix and Table 3) is virtually indistinguishable from the original set (Table 1). The frequency of divergent cases is slightly higher (9.2% to 7.8%), but the difference is not significant ($\chi^2 = 2.07, P > 0.1$). The same holds for more extreme divergence (three or four steps from consensus). The frequency of variable traits is virtually identical (43.4 to 43.1.) Likewise, the difference is not significant when evaluated in terms of the number of divergent traits per species or the distribution of ranges. Nevertheless, there are obvious and highly significant differences between enzymes. Hexoki-

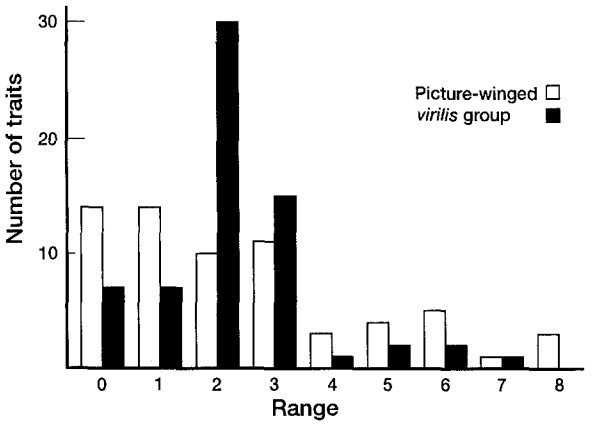

Fig. 2. The distribution of trait ranges. The number of traits is displayed as a function of the range between the lowest and highest activity recorded for each group. Data for the picture-winged group are shown with *open bars* and those for the *virilis* group with *solid bars*. As described in the text, the scale for recording activities (and ranges) utilizes twofold steps, so the widest-recorded range (eight steps) represents an activity difference of 256-fold.

nase and hydroxybutyrate dehydrogenase have high frequencies of divergence, comparable to alcohol dehydrogenase and aldehyde oxidase 1 in the old set; alpha-glycerophosphate dehydrogenase and malate dehydrogenase have intermediate levels, comparable to aldehyde oxidase 2; and aldolase and malic enzyme are relatively conservative, as were octanol dehydrogenase and xanthine dehydrogenase.

Discussion

Species Group Comparisons

The Hawaiian *Drosophila* represent one of the premier examples of explosive speciation and diversification (Carson and Yoon 1982). Several hundred species are known, including about 100 in the picture-winged group. Morphological differences are pronounced and specializations for habitat and host plants are common. Most species are confined to a limited range, often a single volcano. The radiation of most picture-winged species is thought to have occurred within the last five million years (Hunt et al. 1981; Beverley and Wilson 1985; DeSalle and Grimaldi 1991). The *adiastola* subgroup probably diverged earlier, but only four of the 24 species included in this study belong to that group, and dropping them from the analysis makes no appreciable difference in any of the comparisons.

In contrast, the *virilis* group includes only 12 described species. Several have broad, continental distributions and all apparently occupy similar habitats. Morphological differences are subtle, to the extent that there is a history of confusion and dissent regarding which specimens represented distinct species (Throckmorton 1982). Nevertheless, the deepest divergences with the group are thought to have occurred significantly earlier than the major radiation of picture-winged species—probably 10–20 MYA (Spicer 1991). Thus, the *virilis* group, by several criteria, has evolved slowly in comparison to the picture-winged flies.

Our data show that regulatory divergence is significantly more common in the rapidly evolving Hawaiian *Drosophila*. The frequency of divergent cases is about 1.6 times higher. If the preceding age estimates are correct (i.e., divergence within the *virilis* group more ancient than the major picture-winged radiation by a factor of two to four), the rate of accumulation of divergent traits would be at least three to six times greater for species in the latter group. The differences are even more pronounced when one considers more extreme deviation from the consensus. Still, the contrast between the groups is not uniform across enzymes; alcohol dehydrogenase and aldehyde oxidase 1 are highly variable in the picture-winged flies and dramatically less so in the *virilis* group, and they account for most of the overall difference. The obvious caveat is that a different set of enzymes might have given a significantly different picture. However, the second set of enzymes examined in the picture-winged flies yielded results virtually identical to the first set. We also have not sampled either group sufficiently to establish rigorously that divergent traits are fixed. However, we have examined multiple lines of several species in the picture-winged group and most differences appear to be consistent (Dickinson 1980 and unpublished). If that is less often true in the *virilis* group, the difference between the groups would be more pronounced, not less.

Although the rates of evolution of regulatory traits and of morphology are correlated, we cannot claim a causal connection. Indeed, it is likely that we should look to a very different set of genes if we seek regulatory differences that *cause* morphological differences (Dickinson et al. 1993). Even the proposition that the observed regulatory changes are adaptive in some other way (e.g., via altered physiology) is problematical. It may be that both regulatory change and morphological evolution are correlated to other factors. Indeed, certain aspects of the data invite non-adaptive explanations. Isolated populations and frequent founder events almost certainly have been important in the rapid speciation of the Hawaiian *Drosophila*. These same factors might favor chance fixation of variants that have no adaptive value, at least initially. Specifically, Ohta (1976) predicted that near-neutral changes should accumulate more rapidly in small,

Table 3. Summary of conserved and divergent regulatory traits in the picture-winged species group—enzymes with endogenous substrates: the enzymes assayed were aldolase (Ald), alpha-glycerphosphate dehydrogenase (GPDH), D-3-hydroxybutyerate dehydrogenase (HBDH), hexokinase (Hex), malate dehydrogenase (MDH), and malic enzyme (ME)

	Ald	GPDH	HBDH	Hex	MDH	ME	Total
Conserved cases	84	96	88	87	96	101	552
Divergent cases	4	8	16	17	8	3	56
≥3 steps diverged	0	1	5	10	4	0	20
≥4 steps diverged	0	0	2	5	1	0	8
Positive divergence	4	4	12	8	7	2	37
Negative divergence	0	4	4	9	1	1	19

isolated populations, and DeSalle and Templeton (1988) have presented supporting evidence.

Perhaps many changes are associated with speciation per se (Carson and Templeton 1984). In that case, we expect a correlation between frequency of divergent cases and frequency of speciation. We do not know precisely the number of speciation events separating each extant species from a common ancestor of its group, but X in the expression $n = 2^x$ (where n is the known number of species in the group) gives a *minimum* estimate for the group average. That estimate is about 6.5 for the picture-winged group and 3.6 for the *virilis* group. A count of nodes in phylogenetic reconstructions (Carson and Yoon 1982; Spicer 1991) yields comparable figures. The ratio of those numbers (1.8) is close to the ratio of frequencies of divergent regulatory traits in the two groups (1.6). That is consistent with a roughly constant probability of acquiring a divergent trait during each speciation event.

The predominant direction of divergence also is consistent with random fixation. Clark (1991) explored models, based on metabolic control theory, that predict the fitness consequences of altered enzyme levels. He found that fitness functions should be skewed toward higher activities. That is, activity above the optimum leads to a smaller reduction in fitness than does activity below the optimum. Furthermore, this should be reflected in a similarly skewed distribution of activity variants present in a population at equilibrium between mutation and selection. There should be more variants with activities above the optimum than below. These conclusions hold whether selection acts to optimize flux or metabolite level. If chance fixation of near-neutral variants is a major factor in the generation of regulatory divergence, Clark's model leads to the expectation that positive divergence from the consensus would be more common than negative divergence. This follows because, at equilibrium, a larger number of positive variants would be present and available for random fixation. Our data are consistent with this prediction (Tables 1–3). Activity in individual species is higher than the group consensus nearly twice as often as it is below that level (138 to 76). The difference is highly significant ($\chi^2 = 17.9$, $P \ll 0.005$).

Interestingly, alcohol dehydrogenase shows a reverse trend in both data sets. There are 15 high and 30 low variants among picture-winged flies, and two high and six low in the *virilis* group. This deviation from equality is significant for the combined data ($\chi^2 = 8.81$, $P < 0.005$) and for the picture-winged group alone ($\chi^2 = 5$, $P < 0.05$). All other enzymes (including the new set in Hawaiian species) show an excess of positive cases or approximate equality. The basis of this difference is a puzzle for which we cannot at present suggest a solution, but it reinforces the impression that the regulatory systems controlling different enzymes have experienced different selective forces and/or constraints. (See below.)

Finally, the fact that the two groups do not differ significantly either in the number of traits found to be divergent in at least one species or in the distribution of ranges suggests that *virilis* group species are *not* subject to substantially greater constraint. That also seems consistent with chance accumulation of divergent traits, the *virilis* group simply having had fewer "opportunities" to fix variants. However, it also is possible that Hawaiian species more often have experienced selection toward new adaptive optima. Thus, at present we can only be sure that, for these species groups, regulatory diversification correlates with frequent speciation *and* with rapid morphological and ecological diversification. The nature and direction of causality require further clarification.

Enzyme Comparisons

Our primary reason for examining additional enzymes in the picture-winged group was the possibility that the original set was not representative. Several considerations contributed to that concern. A number of authors have looked for patterns in the distribution of polymorphism across structural loci (e.g., Powell 1975; Selander 1976; Ward et al. 1992), and there is some support for a relationship

to metabolic function (Gillespie and Langley 1974). Specifically, enzymes that act on multiple exogenous substrates seem to be more polymorphic than enzymes that act on a single endogenous metabolite. This may reflect greater constraint on the latter. For neutral changes, rates of substitution between species are expected to be proportional to polymorphism within species (Kreitman 1991). The enzymes in our first survey generally fall into the exogenous substrate category and have been reported to be relatively polymorphic (Powell 1975). Moreover, the existence of viable null mutations in *D. melanogaster* indicates that those enzymes are nonessential, at least under laboratory conditions. If these enzymes are subject to less constraint than are enzymes with more central metabolic roles, the extensive regulatory divergence might reflect that fact rather than positive selection. Internal metabolism also seems less likely to change in an adaptive way within a group of closely related organisms than might be the case for factors interacting directly with substances from the environment. Thus, both neutralist and selectionist considerations plausibly lead to the expectation that regulatory divergence might be unusually common for the original set of enzymes.

The enzymes added in this new survey were chosen to contrast in several ways. They are "housekeeping" enzymes whose functions might be needed in most cell types; each is thought to act on a specific product of intermediary metabolism; and all have been reported to display low levels of structural polymorphism in a number of *Drosophila* species (Powell 1975). Hexokinase catalyzes the first step in the glycolytic pathway, and aldolase also is a key enzyme in that pathway. Hydroxybutyrate dehydrogenase catalyzes the reduction of acetoacetate to D-3-hydroxybutyrate in fatty acid metabolism. Malate dehydrogenase catalyzes the formation of oxaloacetic acid from L-malic acid and, thus, plays critical roles in both the tricarboxylic acid cycle and the malate-aspartate shuttle. Finally alpha-glycerophosphate dehydrogenase catalyzes the first step in the glycerol phosphate shuttle.

Our data do not reveal differences between these enzyme sets. As might be expected for "housekeeping" enzymes, the new data reveal fewer tissues lacking detectable activity. Nevertheless, quantitative variation was just as common and virtually as extensive in range. There are highly significant differences between enzymes, but they do not correlate with the suggested classifications. We also see no obvious relationships to any other aspect of metabolic function or to the categories of structural polymorphism suggested by Lewontin (1985). Thus, we agree with Powell et al. (1980), who concluded that the evolutionary forces acting on structural and regulatory diversity are not necessarily correlated. Nevertheless, the differences between individual enzymes suggest that there have been highly significant, if unknown, differences in selective forces, whether favoring change for some enzymes or restricting change for others. Alternatively, new mutations producing altered regulatory patterns may arise at different rates for different genes.

Again, nonadaptive explanations for most of the divergence seem possible. In particular, it is hard to imagine that so many quantitative differences in tissue-specific levels of "housekeeping" enzymes are the result of direct selection. Theoretical considerations suggest that levels of individual enzymes have little effect on net flux through metabolic pathways (Kacser and Burns 1981). Indeed, many systems prove to be resistant even to deliberate efforts to overproduce specific metabolites (Stephanopoulos and Vallino 1991). We have argued elsewhere that, in such circumstances, indirect influences inherent in regulatory networks based on combinatorial mechanisms can drive change that at least is not *directly* adaptive (Dickinson 1988).

Comparisons to gene products other than enzymes may be informative. Several genes that encode transcriptional regulators and signal transduction molecules that regulate the process of segmentation in *Drosophila* embryos are expressed in patterns that are virtually indistinguishable among species of the *virilis* group and, indeed, in *D. melanogaster,* from which they diverged an estimated 60 MYA (Dickinson et al. 1993). Less-extensive examination of similar genes in picture-winged species also gives no indication of rampant regulatory change (unpublished observations). Thus, regulation of this category of genes may be subject to significantly greater constraint than is typical for enzymes. Even so, we cannot conclude that all conserved details are functional. In some cases we already know that certain aspects of the regulation of critical developmental genes are not essential to produce normal morphology or that ectopic expression has no visible effect on development of many tissues (Poole and Kornberg 1988; Bowtell et al. 1989; Casanova and Struhl 1989; Gonzales-Reges and Morata 1990; Ruvkun et al. 1991). Of course, features that are not essential can still be functional and subject to selection (Zuckerkandl 1992). In addition, constraint operating on *some* important features in a tightly integrated regulatory network may *indirectly* lead to conservation of details that are not themselves essential or even functional (Dickinson 1988).

Even for the genes encoding enzymes that are the primary focus of this paper, the majority of regulatory traits are conserved at the level of our anal-

ysis. In many cases, these conserved features include tissues with relatively high enzyme activities. By analogy to conserved regions in structural genes, conserved sites of expression may reveal locations of useful functions. However, rates of accumulation of selectively neutral variants should be proportional to mutation rates, and we have virtually no information on the frequency of new mutations that significantly alter regulatory patterns. Thus, we cannot be certain whether conserved traits reflect selective elimination of variants or absence of relevant mutations. Indirect selection, as mentioned in the preceding paragraph, also may be a factor. Clearly, much remains to be done before we will have anything approaching a general picture of the force shaping regulatory evolution.

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Appendix

Consensus expression levels (see main text) for five enzymes in 12 species of the *virilis* species group and for six enzymes in eight species of the picture-winged group are tabulated. A group consensus for each trait also is given and individual cases that diverge from that consensus by more than ± 1 twofold step are bold and underlined. The first six columns are larval tissues as follows: carcass (LC), midgut (LM), hindgut (LH), Malpighian tubules (LT), fat body (LF), and salivary gland (LS). The next seven are adult tissues: head (AD), midgut (AM), hindgut (AH), Malpighian tubules (AT), empty abdomen (AA), ovaries (AO), and male reproductive tract (AR). The final column lists the total number of divergent traits for each species. The appendix to a previous paper (Dickinson 1980) contains similar data for the first five enzymes in species of the picture-winged group that are summarized in Table 3 and compared to these data in the main text.

Alcohol dehydrogenase

	LC	LM	LH	LT	LF	LS	AD	AM	AH	AT	AA	AO	AR	Div
texana	5	5	1	2	6	0	7	**5**	3	0	7	2	1	1
novamexicana	6	5	1	**3**	6	0	5	1	**0**	0	5	0	0	2
virilis	5	5	1	1	7	0	7	3	3	0	7	1	2	0
lummei	6	5	1	1	6	0	5	2	2	0	5	0	0	0
americana	5	5	2	2	6	0	5	3	1	0	6	0	0	0
littoralis	5	4	0	1	6	0	5	2	2	0	5	1	0	0
ezoana	5	**2**	0	0	5	0	5	3	3	0	5	1	2	1
lacicola	5	4	0	1	5	0	5	1	1	0	**4**	0	1	1
montana	6	5	1	1	6	0	5	2	1	0	6	1	0	0
borealis	6	5	0	2	7	0	5	**0**	**0**	0	5	1	0	2
kanekoi	**3**	3	0	2	5	0	7	3	3	0	7	1	0	1
flavomontana	6	4	1	1	7	0	5	3	2	0	6	1	0	0
Group consensus	5	4	1	1	6	0	6	2	2	0	6	1	1	

Aldehyde oxidase 1

	LC	LM	LH	LT	LF	LS	AD	AM	AH	AT	AA	AO	AR	Div
texana	3	1	4	0	2	0	9	3	**7**	**3**	7	9	7	2
novamexicana	**6**	1	3	2	2	0	7	2	5	1	8	8	6	1
virilis	3	1	**6**	0	2	0	9	1	3	0	7	7	6	1
lummei	3	0	3	0	2	0	7	**0**	**6**	0	6	9	6	2
americana	**8**	2	**6**	0	2	0	8	2	4	0	7	8	6	2
littoralis	—	—	—	—	—	—	—	—	—	—	—	—	—	—
ezoana	**8**	0	**8**	0	3	1	8	3	**2**	**3**	6	7	**8**	5
lacicola	2	0	3	0	3	0	8	2	3	1	6	**4**	5	1
montana	1	0	2	0	3	0	8	2	5	1	7	7	6	0
borealis	1	1	3	0	**0**	0	8	2	5	1	**5**	**10**	5	3
kanekoi	**8**	1	3	0	4	0	8	2	**7**	2	7	8	**8**	3
flavomontana	2	2	2	0	4	0	8	2	3	**3**	8	**6**	5	2
Group consensus	2	1	3	1	3	0	8	2	4	1	7	8	6	

Aldehyde oxidase 2

	LC	LM	LH	LT	LF	LS	AD	AM	AH	AT	AA	AO	AR	Div
texana	0	3	0	0	0	0	0	4	0	2	2	0	3	0
novamexicana	0	4	0	2	1	0	0	5	0	2	1	0	3	0
virilis	0	4	0	1	1	0	0	6	0	0	2	0	2	0
lummei	0	4	0	0	0	0	0	4	0	0	1	1	3	0
americana	0	5	1	0	1	0	0	4	1	2	2	1	3	0
littoralis	—	—	—	—	—	—	—	—	—	—	—	—	—	—
ezoana	0	4	0	2	0	0	0	5	1	1	1	0	4	0
lacicola	0	3	0	0	0	0	0	**3**	0	1	0	0	2	1
montana	0	3	0	1	0	0	0	4	0	0	1	0	2	0
borealis	1	4	0	1	0	0	1	5	1	0	2	1	2	0

kanekoi	0	4	1	1	1	0	0	6	0	1	2	0	3	0
flavomontana	0	4	2	1	2	0	2	6	1	2	2	0	3	0
Group consensus	0	4	1	1	1	0	1	5	0	1	1	0	3	

Octanol dehydrogenase

	LC	LM	LH	LT	LF	LS	AD	AM	AH	AT	AA	AO	AR	Div
texana	3	3	1	2	5	0	3	3	1	1	4	6	3	0
novamexicana	4	3	1	2	5	0	3	2	0	0	3	5	3	0
virilis	4	3	2	2	5	0	3	3	1	1	4	6	4	0
lummei	3	4	2	2	5	0	2	2	0	0	3	4	3	0
americana	2	3	2	3	5	0	4	3	1	1	4	4	3	0
littoralis	3	3	1	2	5	0	3	2	0	1	3	4	3	0
ezoana	3	3	1	2	4	0	2	2	0	0	2	4	3	0
lacicola	2	5	1	2	4	0	2	3	0	1	3	4	3	0
montana	3	3	1	2	5	0	2	1	0	0	3	4	2	0
borealis	2	3	0	2	5	0	2	2	0	0	3	4	3	0
kanekoi	**5**	4	1	2	5	0	3	3	1	2	4	6	4	1
flavomontana	4	4	1	**0**	4	0	3	3	1	1	3	6	4	1
Group consensus	3	4	1	2	5	0	3	2	0	1	3	5	3	

Xanthine dehydrogenase

	LC	LM	LH	LT	LF	LS	AD	AM	AH	AT	AA	AO	AR	Div
texana	2	3	0	0	4	0	—	4	—	2	6	0	3	0
novamexicana	2	3	0	**3**	4	0	2	3	1	1	**3**	0	3	2
virilis	2	3	0	0	5	0	3	**5**	0	3	4	0	3	1
lummei	3	4	0	0	5	0	3	3	0	1	5	0	3	0
americana	1	2	0	**3**	5	0	2	3	1	1	**3**	0	3	2
littoralis	2	2	0	0	5	0	3	2	0	1	—	0	3	0
ezoana	1	2	0	1	4	0	2	2	1	1	5	0	3	0
lacicola	2	2	0	0	3	0	3	3	0	1	5	0	2	0
montana	2	2	0	1	5	0	2	4	0	1	4	0	2	0
borealis	2	3	0	2	5	0	4	3	0	1	6	0	3	0
kanekoi	2	2	0	2	4	0	3	4	1	3	6	0	4	0
flavomontana	2	3	0	1	5	0	3	2	0	2	5	0	3	0
Group consensus	2	3	0	1	4	0	3	3	0	2	5	0	3	

Aldolase

	LC	LM	LH	LT	LF	LS	AD	AM	AH	AT	AA	AO	AR	Div
heteroneura	2	1	0	2	3	0	4	—	—	2	5	2	2	0
silvestris	2	0	0	1	3	0	3	—	—	1	5	2	1	0
crucigera	3	**3**	0	1	3	0	5	—	—	0	**6**	2	2	2
grimshawi	2	1	0	1	2	0	3	—	—	0	3	3	2	0
affinidisjuncta	3	1	1	0	1	1	3	—	—	1	4	2	2	0
hirtipalpis	2	1	0	0	3	0	4	—	—	0	4	2	1	0
silvarentis	4	**3**	2	2	3	2	4	—	—	2	4	3	2	1
hawaiiensis	3	0	0	0	**4**	0	4	—	—	2	5	3	1	1
Group consensus	3	1	1	1	2	1	4	—	—	1	4	2	2	

Alpha-glycerophosphate dehydrogenase

	LC	LM	LH	LT	LF	LS	AD	AM	AH	AT	AA	AO	AR	Div
heteroneura	6	5	3	2	6	3	7	5	5	4	6	4	5	0
silvestris	6	4	**0**	1	5	3	7	6	4	3	6	4	4	1
crucigera	7	4	1	1	6	3	6	4	3	2	5	4	**2**	1

grimshawi	6	4	2	2	6	**6**	7	5	4	3	6	5	4	1
affinidisjuncta	6	3	**0**	**4**	5	5	7	5	4	4	6	4	4	2
hirtipalpis	6	5	3	0	6	5	8	5	4	3	6	5	4	0
silvarentis	6	4	2	0	**8**	4	8	6	4	2	**8**	5	5	2
hawaiiensis	5	4	2	1	5	4	7	5	5	**1**	7	4	4	1
Group consensus	6	4	2	1	6	4	7	5	4	3	6	4	4	

D-3-hydroxybutyrate dehydrogenase

	LC	LM	LH	LT	LF	LS	AD	AM	AH	AT	AA	AO	AR	Div
heteroneura	3	**4**	1	5	4	1	6	**5**	2	6	6	2	2	2
silvestris	3	**0**	1	**6**	5	0	6	**6**	2	**7**	7	2	2	4
crucigera	3	**0**	0	3	3	**3**	4	2	0	4	6	2	1	2
grimshawi	3	1	1	4	3	**3**	5	2	1	4	7	3	1	1
affinidisjuncta	4	1	0	3	3	0	5	1	0	5	7	3	2	0
hirtipalpis	2	3	**3**	3	4	**4**	5	**4**	0	6	7	3	1	3
silvarentis	3	3	0	**1**	**6**	0	4	2	0	**1**	6	2	0	3
hawaiiensis	3	3	2	5	5	**3**	6	3	1	6	7	1	1	1
Group consensus	3	2	1	4	4	1	5	2	1	5	6	2	1	

Hexokinase

	LC	LM	LH	LT	LF	LS	AD	AM	AH	AT	AA	AO	AR	Div
heteroneura	5	6	0	**0**	**3**	**0**	8	6	**4**	**7**	8	5	5	5
silvestris	7	4	1	3	5	5	8	5	3	**6**	8	6	5	1
crucigera	6	**2**	0	4	6	**2**	7	6	3	3	7	6	3	2
grimshawi	7	4	0	**1**	5	4	7	5	3	**6**	8	6	5	2
affinidisjuncta	7	6	1	3	7	4	7	5	3	3	7	6	4	0
hirtipalpis	7	5	0	3	6	3	6	6	2	1	8	5	4	0
silvarentis	**8**	**7**	1	**0**	7	**6**	7	**8**	1	2	8	6	3	5
hawaiiensis	6	**1**	0	**0**	6	4	7	6	3	1	7	5	3	2
Group consensus	6	5	0	3	6	4	7	6	2	2	8	5	4	

Malate dehydgrogenase

	LC	LM	LH	LT	LF	LS	AD	AM	AH	AT	AA	AO	AR	Div
heteroneura	13	11	7	8	12	7	12	11	7	8	12	9	**10**	1
silvestris	12	10	6	8	12	**4**	12	**12**	**9**	**10**	13	11	**10**	5
crucigera	12	10	6	7	11	7	11	10	6	6	11	**12**	7	1
grimshawi	13	10	7	8	12	9	12	11	8	7	12	11	7	0
affinidisjuncta	13	10	6	7	12	8	11	11	8	8	13	**12**	8	1
hirtipalpis	12	10	5	7	11	7	10	10	7	7	11	9	7	0
silvarentis	12	9	6	8	11	8	10	9	6	7	11	9	7	0
hawaiiensis	12	10	7	8	11	8	11	10	7	8	12	10	7	0
Group consensus	12	10	6	8	11	8	11	10	7	7	12	10	7	

Malic enzyme

	LC	LM	LH	LT	LF	LS	AD	AM	AH	AT	AA	AO	AR	Div
heteroneura	8	9	6	8	12	5	10	9	7	9	12	9	9	0
silvestris	9	9	7	8	10	6	11	9	8	10	12	10	**10**	1
crucigera	10	10	6	7	11	7	10	7	7	9	10	9	8	0
grimshawi	8	9	5	7	11	5	9	7	6	8	11	9	8	0
affinidisjuncta	10	10	5	6	11	5	9	**6**	6	8	11	9	8	1
hirtipalpis	9	8	6	6	11	7	10	7	7	8	10	8	7	0
silvarentis	9	9	6	6	11	6	11	9	8	9	12	9	9	0
hawaiiensis	10	8	6	8	11	7	**12**	7	8	9	12	9	8	1
Group consensus	9	9	6	7	11	6	10	8	7	9	11	9	8	