A Comparison of Phylogenies Based on Structural and Tissue-Expressional Differences of Enzymes in a Family of Teleost Fishes (Salmoniformes: Umbridae)\textsuperscript{1}

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Differences in tissue patterns of enzyme-locus expression were used to infer the extent of divergence among species. Enzyme activities, at 27 loci in six tissues of 15 individuals in four species of Umbridae (Salmoniformes), were estimated on the basis of starch-gel electrophoresis of tissue extracts subjected to twofold serial dilutions and subsequent histochemical staining to determine the visual endpoints. Some locus-tissue expressions diverged markedly among species, while other locus-tissue expressions were conserved. Differences in activity for a given enzyme among tissues, as well as for different enzymes within the same tissue, were sufficiently independent to permit each locus-tissue expression of a species to be treated as a separate character. Statistically significant differences in levels of tissue-enzyme activities among species were then used to construct a phylogeny. The phylogeny constructed using tissue-enzyme expressional differences was similar to that based on enzyme structural differences (genetic distances) and to one of the morphologically based phylogenies. This congruence suggests that species differences in tissue patterns of enzyme-locus expression can be used to test a variety of systematic and evolutionary hypotheses.

Introduction

Divergence in the regulation of the timing and level of expression of structural genes appears to play a more important role in organismic evolution than does divergence in the structural genes being regulated (Zuckerkandl and Pauling 1965; King and Wilson 1975; Markert et al. 1975; Wilson 1976; Ferris and Whitt 1979; Dickinson 1980; King and McDonald 1983). The manifestations of evolutionary changes in gene expression can be observed at many different organismic levels, ranging from modifications in intracellular concentrations of macromolecules to alterations in gross morphology (Raff and Kaufman 1983). In the context of the present study, the gene-expressional differences that we are investigating are the assumed steady-state levels of enzymes in various differentiated adult tissues. Alterations of this facet of gene expression could be brought about by genetic changes at any of many levels affecting enzyme synthesis or catabolism, presumably resulting in most instances from changes at gene(s) other than those coding for the enzyme structure.

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Species differences in the tissue patterns of regulation of enzymes and isozymes have proved to be evolutionarily informative characters for inferring the extent of gene-expressional divergence among species and/or for resolving organismic phylogenies (Syner and Goodman 1966; Shows et al. 1969; Markert et al. 1975; Zuckerkandl 1978; Shaklee and Whitt 1981; Whitt 1983; Buth 1984). As pointed out by Wilson et al. (1977), greater than tenfold differences in levels of selected proteins often have been found between different species. Quantification of differences in enzyme-locus expressions for catostomid fishes (Ferris and Whitt 1979), Hawaiian picture-winged Drosophila (Dickinson 1980), and higher plants (Gottlieb 1982) were some of the first comprehensive studies measuring inferred gene-expressional differences within and between species and then using these differences to test hypotheses about their systematic relationships.

Rationale for the Experimental Approach

Gene-expressional differences within and among species were inferred from the quantitation of differences in tissue expression of enzyme and isozyme loci. Quantitation of variation among individuals in activity levels of enzymes and isozymes (within and among tissues) was based on the relative levels of histochemically visualized enzyme activity after starch-gel electrophoresis of tissue extracts subjected to serial dilutions. Serial dilution procedures to visual endpoints provide a highly repeatable means of comparing enzyme activities (Klebe 1975; Ferris and Whitt 1979; Dickinson 1980).

In the present study, a relatively depauperate family of fishes, the mudminnows (Umbridae, Salmoniformes) was chosen as the taxon to be investigated. We were able to investigate four of the five species. The small number of species studied allowed us to increase the number of enzyme loci, tissues, and individuals sampled from each species. The Umbridae contains both recently derived species and more anciently derived species. The umbrids are a cohesive group occupying similar habitats (Cavender 1969; Nelson 1972) and possess a reasonably detailed phylogeny based on morphology and fossil evidence (Cavender 1969; Nelson 1972; Wilson and Veilleux 1982), karyology (Beamish et al. 1971; Ráb 1981; Banarescu et al. 1983), and genetic distance based on 51 loci (Kettler and Whitt 1984; Kettler 1985; M. K. Kettler and G. S. Whitt, unpublished data). In addition, the earlier observation of a rather marked tissue-enzyme regulatory divergence among the umbrid species at one enzyme locus (Ldh-C) (Kettler and Whitt 1986) suggested that increasing the number of enzyme loci and tissues sampled would enable us to determine whether tissue expressions for a series of enzyme loci can be pooled to obtain, between species, a gene-expressional distance analogous to the genetic distance based on pooled enzyme-locus structural differences.

Material and Methods

The activities of enzymes encoded by 27 loci in each of six tissues (brain, eye, heart, liver, muscle, and stomach) were determined for each of 15 individuals from each of four species of Umbridae using the Klebe serial dilution procedure (Klebe 1975). Reproductively mature individuals were collected in the field and immediately frozen on dry ice. Collection localities for each species were as follows: Umbra limi, Buffalo River, Buffalo Co., Wis.; Umbra pygmaea, Smith Creek, Hanover Co., N.C.; Dallia pectoralis, Chena River, Yukon-Kuskokwim Co., Alaska; and Novumbra hubbsi, Connor Creek, Grays Harbor Co., Wash.). Specimens were stored at -20 C for no more than 6 mo prior to electrophoresis. For each of the four species, a sample of individuals was surveyed repeatedly over a 6-mo period for selected enzymes and
Gene-expression differences among species showed a decline in activity during storage of no greater—and usually less—than the twofold level detectable by the serial dilution procedure.

Tissues were weighed at 4°C, and 1 ml of 0.1 M Tris-HCl, pH 7.0, was added per 0.2 g tissue. All tissues were homogenized for 15–20 s at 4°C with a motorized Potter-Elvehjem pestle. Slight variations in the length of time that the tissues were homogenized did not appreciably increase or decrease the enzyme activity observed. After centrifugation at 23,500 g for 20 min at 4°C, a series of twofold dilutions of each supernatant were placed into equal-sized slots (40 μl) on the same starch gel. Vertical starch-gel electrophoresis (Buchler Apparatus, Nuclear Chicago Corp., Fort Lee, N.J.) was carried out for 18 h. Electrophoretic conditions are described in detail by Kettler (1985) and M. K. Kettler and G. S. Whitt (unpublished data). The enzymes, Enzyme Commission numbers, and locus designations are listed in Table 1.

The relative levels of each enzyme activity in a tissue were determined by means of the Klebe (1975) serial dilution technique. Klebe (1975) demonstrated that, given a particular enzyme activity, the last band detected on electrophoresis and exhaustive staining of twofold dilutions of that enzyme (the visual endpoint) always occurred at the same dilution. The relative and absolute activity of a given enzyme could therefore be precisely measured. To verify the repeatability of this technique in the present

| Enzyme                                      | Locus Designation | Enzyme Commission No. *
<table>
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<td>Aconitate hydratase</td>
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<tr>
<td></td>
<td>Ck-C</td>
<td></td>
</tr>
<tr>
<td>Dihydrolipoamide reductase</td>
<td>Dlr-A</td>
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<td>Dlr-B</td>
<td></td>
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<td>Fructose-bisphosphatase</td>
<td>Fbp-A</td>
<td>3.1.3.11</td>
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<td>Glucosephosphate isomerase</td>
<td>Gpi-A</td>
<td>5.3.1.9</td>
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<td></td>
<td>Gpi-B</td>
<td></td>
</tr>
<tr>
<td>Glycerol-3-phosphate dehydrogenase</td>
<td>G3pdh-A</td>
<td>1.1.1.8</td>
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<td>G3pdh-B</td>
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<td>Lactate dehydrogenase</td>
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<td>Ldh-C</td>
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<td></td>
<td>s-Mdh-B</td>
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<td>Malic enzyme (NADP-dependent MDH)</td>
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<td>s-Me-A</td>
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<tr>
<td>Phosphoglucomutase</td>
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</table>

study, multiple samples of a single tissue from a single individual were electrophoresed on different gels. The visual endpoints for these multiple samples were almost always the same, with no greater than a twofold variability in visual endpoints ever being observed. The use of serial twofold dilutions reduces the probability of detecting small activity differences caused either by experimental error or by small differences in the kinetic properties of an enzyme. When multiple samples of a single tissue extract from a given individual were homogenized separately and then each series of dilutions electrophoresed (on the same or different gels), identical visual endpoints were obtained. Thus, any variation in the homogenization procedure was below the threshold of detectability of the twofold serial dilution. Although highly repeatable, the histochemical staining method employed for multilocus isozymes is not an exact measure of the activity of each isozyme because each isozyme has a different optimal pH and substrate concentration. Presumably this would represent a relatively constant bias for the same enzyme from different tissues and from different individuals.

The number of dilutions that were required to reach the visual endpoint were determined for each enzyme in each of the six tissues examined. Identical visual endpoints imply similar but not necessarily identical initial activity levels. We have, therefore, treated the numbers of twofold dilutions to visual endpoints as having reliable ordinal-scale accuracy only, the assumption of continuous-scale precision being clearly unwarranted.

Tissue independence and enzyme independence were tested using Goodman and Kruskal's (1954) gamma coefficient as a superior measure of rank-order correlation in contingency tables (Ghent 1984). Samples of 15 fish from each of the four species permitted four distributions of 15 visual endpoints to be tested for the presence of any significant differences using the Kruskal-Wallis (Kruskal and Wallis 1952) H-test, a nonparametric analysis of variance by ranks. Where significant H-tests were obtained, the Newman-Keuls post hoc procedure (Newman 1939; Keuls 1952; Zar 1984) was employed to determine which pairwise species comparisons were significantly different. A second post hoc procedure, the more conservative Dunn's test (Dunn 1964; Hollander and Wolfe 1973), was separately applied in the same manner. These procedures permitted totals of 27 (loci) × 6 (tissues) = 162 locus-tissue comparisons to be scored for significance for each of the six pairwise species comparisons. The percentages of significant comparisons in these six sets of 162 comparisons were then used as distances between species pairs in constructing separate phenograms for the Newman-Keuls and the Dunn's post hoc tests.

The computational complexity of this new statistical approach to phenogram construction is constant for given numbers of loci and tissues, and it increases with increasing numbers of species only as these effect slight increases in the labor of the individual Kruskal-Wallis H-tests and the ensuing post hoc procedures. The Newman-Keuls procedure can be applied only where all species are represented by identical sample sizes. While Dunn's procedure permits varying sample sizes, its computation is simplified where all sample sizes are the same.

An extension of the foregoing analysis employed the Poisson distribution to estimate the number of detectable changes in gene expression that presumably would have occurred to obtain the total number of gene-expressional differences observed for each species pair. If \( d_{ij} \) is a Newman-Keuls value taken from the \( i \)th column and \( j \)th row of table 4, then \( (100 - d_{ij})/100 = e^{-r} \), where \( r \) = the number of expression changes for each tissue-enzyme. This equation can then be rearranged to give \( r = \ln (1 - d_{ij}/100) \). The branch lengths of a tree constructed using this method of
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analysis are then proportional to the estimated number of expressional changes per tissue-enzyme rather than to the number of expressional differences observed. This Poisson extension brings out rate differences that are pictorially suppressed in the initial nonparametric analysis.

Results

All adult individuals sampled within a species tended to have the same or similar visual endpoints for any given enzyme in a given tissue, although exceptions were observed. The complete data matrices (27 pages of tables) of the visual endpoints of enzyme activity for each of 27 loci, in each of six tissues, in each of 15 individuals in each of four species are available directly from the senior author. Where differences between individuals did exist, they were usually no greater than twofold. In a few cases the tissue-enzyme activity within a species was quite variable. For example, PGI-A2 in the muscle of Novumbra hubbsi ranged from 1/4 to 1/128 dilution to visual endpoint in the two most extreme individuals in a population.

Where enzyme activity variation was observed within species, some tissues were more variable than others and some enzymes were more variable than other enzymes. Most increases or decreases in activity for an enzyme in a tissue were independent of the activity in other tissues. The level of variation within a species was quite low compared to the between-species differences in tissue-enzyme activity. In almost every case, the median visual endpoint was the same as the modal visual endpoint for a species. The median visual endpoints for each species for each tissue are shown in table 2.

Before the tissue-enzyme activity differences and similarities for the four species could be compared statistically, the extent of interaction among tissues and among enzymes had to be determined. The interdependence of these variables was tested using Goodman and Kruskal's (1954) gamma coefficient, as well as its test of significance, identical to that of tau (Ghent 1976). In general, tissue-enzyme activities were found to be independent of each other, with increases or decreases in activity of an enzyme in one tissue generally independent of the changes in that enzyme's activity in other tissues (table 3). Of 927 tissue comparisons, only 31 (3.34%) yielded nominally significant coefficients at $P = 0.05$ (or slightly less than the random [5%] expected frequency). In one of the three cases in which the same tissues were correlated for different species (in all three cases the Umbra species), the direction of correlation was the same, being negative for both species. In the remaining two cases the common significant correlations were opposite in direction.

Enzymes within a tissue were also found to be independent of each other, with increases or decreases in activity of different enzymes within a given tissue generally not correlated with each other. It was not possible to test all possible enzyme associations, but selected enzymes within each of a variety of categories were tested for possible correlations of activity increases or decreases within single tissues for each species. The following paired categories were tested for correlated responses: citric acid cycle enzymes versus citric acid cycle enzymes, glycolytic enzymes versus glycolytic enzymes, mitochondrial enzymes versus mitochondrial enzymes, multilocus isozymes versus multilocus isozymes, tissue-restricted enzymes versus generally expressed enzymes, tissue-restricted enzymes versus tissue-restricted enzymes, structurally polymorphic enzymes versus nonpolymorphic enzymes, and monomeric enzymes versus polymeric enzymes. Of the total 1,038 paired enzyme comparisons that were tested
<table>
<thead>
<tr>
<th>LOCUS*</th>
<th>Umbra limi</th>
<th>U. pygmaea</th>
<th>Dallia pectoralis</th>
<th>Novumbra hubbi</th>
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<tr>
<td></td>
<td>B</td>
<td>E</td>
<td>H</td>
<td>L</td>
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</tr>
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NOTE.—B = brain; E = eye; H = heart; L = liver; M = muscle; and S = stomach.
* See Table 1 for enzyme abbreviations.
### Table 3

**Significant Correlations of Tissue Pairs for 27 Loci as Determined by the Kendall-Tau Test for Rank-Order Correlation**

<table>
<thead>
<tr>
<th>TISSUE PAIRS</th>
<th>Umbra limi</th>
<th>U. pygmaea</th>
<th>Novumbra hubbsi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain-Eye</td>
<td>-Gpi-A (0.018)</td>
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<td>+Gpi-B (0.036)</td>
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<tr>
<td>Brain-Heart</td>
<td>-Ck-A (0.002)</td>
<td>+Ck-C (0.028)</td>
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<tr>
<td>Brain-Liver</td>
<td>-Gpi-A (0.027)</td>
<td>-Gpi-A (0.038)</td>
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<tr>
<td>Brain-Muscle</td>
<td>+Gpi-A (0.012)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain-Stomach</td>
<td>-Ck-C (0.003)</td>
<td></td>
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<tr>
<td>Eye-Heart*</td>
<td>-Pgm-A (0.017)</td>
<td></td>
<td>-G3pdh-B (0.018)</td>
</tr>
<tr>
<td>Eye-Muscle*</td>
<td>-Gpi-A (0.006)</td>
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<tr>
<td>Heart-Liver</td>
<td>+Gpi-B (0.043)</td>
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<td>-Gpi-A (0.003)</td>
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<tr>
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<td>+Ck-C (0.014)</td>
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<td>-Ck-c (0.002)</td>
<td>-Gpi-A (0.006)</td>
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**NOTE.**—There were no significant correlations for *Dallia pectoralis* for any tissue pairs. Positive and negative correlations are indicated by + and −; the significance of the correlation is shown in parentheses. *No significant correlations for any species.

within species and within tissues, only five pairs of enzymes were statistically significant in their correlation: phosphoglucomutase-A and glucosephosphate isomerase-A isozymes in *U. limi* muscle tissue (P ≤ 0.0004); glucosephosphate isomerase-A and glucosephosphate isomerase-B isozymes in *U. limi* stomach tissue (P ≤ 0.001); m-malic enzyme and s-aconitase in *U. pygmaea* liver tissue (P ≤ 0.006); creatine kinase-A and creatine kinase-C isozymes in *U. pygmaea* heart tissue (P ≤ 0.007); and glycerol-3-phosphate dehydrogenase-A and glycerol-3-phosphate dehydrogenase-B isozymes in *N. hubbsi* brain tissue (P ≤ 0.03). Four of the five significant correlations were related to the glycolytic pathway (*Pgm-A vs. Gpi-A, Gpi-A vs. Gpi-B, Ck-A vs. Ck-C, and G3pdh-A vs. G3pdh-B*), and one comparison was related to the citric acid cycle (*m-Me-A vs. s-Acn-A*). Four of the five comparisons involved multilocus isozymes,
and two comparisons involved a monomer versus polymer comparison (Pgm-A vs. Gpi-A and s-Acn-A vs. m-Me-A). There were no significant correlations involving mitochondrial versus mitochondrial comparisons, mitochondrial versus cytosolic forms of the same enzyme, or either tissue-restricted versus tissue-restricted or polymorphic versus nonpolymorphic comparisons. No pattern to the significant correlations was observed, in regard to either tissues or species involved; that is, a significant correlation for a given enzyme pair in one tissue of a species was not paralleled by either a similar difference in other tissues of the same species or by a similar correlation within a tissue among all species.

With so few enzyme-enzyme and tissue-tissue correlations detected, the data were treated as satisfactorily independent for Kruskal-Wallis (Kruskal and Wallis 1952) H-testing and the ensuing post hoc multiple-comparison procedures. The results of each of the 972 pairwise species comparisons (for a tissue and enzyme locus) for each of the post hoc procedures are available from the senior author. Substantially fewer significant differences were detected with Dunn’s (1964) conservative procedure, but all comparisons that were significant for the Dunn procedure were also significant for the Newman-Keuls procedure.

The percentage of significantly different species comparisons are shown in table 4 for both the Newman-Keuls (below the diagonal) and the Dunn (above the diagonal) post hoc procedures. The fewest statistical differences in enzyme activity for a pairwise species comparison were for the congeners U. limi and U. pygmaea. The next fewer statistical differences for a pairwise species comparison were between Dallia and Novumbra genera.

The phenetic trees that were constructed using the results of the Dunn procedure and the Newman-Keuls procedure are shown in figure 1. These gene-expression trees did not differ in branching pattern, but they did differ in branch length, because the Newman-Keuls procedure detected more significant differences than did the Dunn procedure.

The values for the Poisson procedure are shown in table 5, and the tree constructed from this analysis is shown in figure 2. Values obtained by summing the branch lengths of this tree are shown in table 5. The agreement between the two halves of table 5 suggests that the analysis was robust and that the Poisson tree (fig. 2) is more descriptive of the data than are the trees shown in figure 1. The tree derived from the Poisson analysis offered information that was suppressed in our first analysis, since the divergence points in the first trees are actually average distances. The second analysis also corrected for multiple changes in a single tissue enzyme. Multiple changes can make pairs of distances seem more similar than they actually were.

Table 4
Gene-Expression Distance Matrix Derived from the Newman-Keuls and Dunn Procedures

<table>
<thead>
<tr>
<th></th>
<th>Umbra limi</th>
<th>U. pygmaea</th>
<th>Dallia pectoralis</th>
<th>Novumbra hubbsi</th>
</tr>
</thead>
<tbody>
<tr>
<td>U. limi</td>
<td>—</td>
<td>6.2%</td>
<td>60.5%</td>
<td>62.4%</td>
</tr>
<tr>
<td>U. pygmaea</td>
<td>26.5%</td>
<td>—</td>
<td>63.0%</td>
<td>59.9%</td>
</tr>
<tr>
<td>D. pectoralis</td>
<td>66.7%</td>
<td>69.1%</td>
<td>—</td>
<td>41.4%</td>
</tr>
<tr>
<td>N. hubbsi</td>
<td>75.3%</td>
<td>77.2%</td>
<td>66.1%</td>
<td>—</td>
</tr>
</tbody>
</table>

NOTE.—Distances obtained using the Dunn procedure are shown above the diagonal. Distances obtained using the Newman-Keuls procedure are shown below the diagonal.
Discussion

The umbrid species differences observed in tissue patterns of gene expression could be caused by alterations at one or more of a series of cellular levels, e.g., transcription, processing, translation, or catabolism. The observation that variation in enzyme activity was generally tissue and enzyme independent suggests that whatever the mechanism(s) involved, it was exerting fairly enzyme-specific and tissue-specific changes and was not exerting "global" changes affecting either many enzymes of the same tissue or the same enzyme in many tissues.

It is conceivable that a portion of the within-species variation in tissue-enzyme activities may have been contributed by polymorphic enzyme loci whose allelic isozymes possess significant functional differences (Koehn 1969; Powers et al. 1983; Watt 1985). However, we have observed a number of enzyme polymorphisms, and whenever heterozygous individuals were subjected to the Klebe dilution procedure, the allelic
variants always had the same visual endpoints, suggesting that any kinetic differences that might have existed between the allelic isozymes were below the twofold difference that the Klebe dilution procedure detected.

When between-species comparisons were made of the activity of an orthologous enzyme, the difference in its activity in one tissue was generally statistically independent of differences in its activity in another tissue. This tissue-independent activity divergence tends to exclude the possibility that the between-species differences in activity are due primarily to species differences in the kinetic properties of orthologous enzymes. Species differences in enzyme activity due to a marked change in the structure and function of an enzyme would be expected to result in a coordinate change in activity for that enzyme in all the tissues in which it is synthesized. Furthermore, the tissue-enzyme activity differences between umbrid species are often much greater than can be accounted for by kinetic differences between orthologous enzymes in different species (Pesce et al. 1967; Yancey and Somero 1978); for example, although slight kinetic differences exist for orthologous lactate dehydrogenase isozymes in different species, the kinetic properties are fairly conserved over broad taxonomic groups, with differences falling within fairly narrow bounds (Yancey and Somero 1978).

The enzyme locus-expression differences between species that were measured by the Klebe dilution procedure may have been caused by a single mutation or by many mutations at any of a number of loci that ultimately affect the levels of a specific enzyme. Our observation that the differences in gene expression within a species of umbrid were generally rather small whereas the differences in tissue-enzyme activity between species were usually greater is consistent with the hypothesis that an accumulation of mutations affects the regulation of steady-state enzyme levels. Furthermore, the numbers of enzymes and tissues affected are greater between species than they are between individuals within a species. The marked species differences in the tissue expressions of certain isozymes, e.g., lactate dehydrogenase C4, suggest that the partial functional redundancies of isozymes and the overlapping of their cellular and tissue expression provide increased opportunities for evolutionary divergence of gene-expression patterns without as great a selective impact as one might expect for single-gene enzyme systems.

Some enzyme expressions were more divergent than others over the same period of evolutionary time. Different rates of change in gene expression for different enzymes would be analogous to the different rates of change in the structures of different enzymes that are used for genetic-distance studies (Sarich 1977). The observation that increasing the number of loci sampled can be more informative than increasing the number of individuals in genetic-distance studies (Nei 1978) is probably also applicable to studies of differences in gene expression, with additional benefits being derived from increasing the number of tissues sampled. Also analogous with genetic distances, the gene-expression distances would be expected to reach a saturation point after longer divergence times.
A comparison of the genetic distances and the gene-expressional distances among these species has been made within the context of the fossil record. The divergence of *Dallia* and *Novumbra* appears to have occurred at least 25–30 Myr ago, since *N. oregensis* is known from the Oligocene and a "*Dallia* sp." is known from the Miocene (Nelson 1972). Gene-expressional divergence for these species ranged from 41% (Dunn) to 66% (Newman-Keuls), compared to their modified Rogers genetic distance (Wright 1978) of 0.84 (Kettler and Whitt 1984; Kettler 1985; M. K. Kettler and G. S. Whitt, unpublished data). On the basis of the fossil record alone, the divergence of the *Umbra* species from the *Dallia-Novumbra* genera may have occurred as much as 33–36 Myr ago (Nelson 1972; Sytchevskaya 1976). The gene-expressional divergence observed for the *Umbra* species versus *Dallia* and *Novumbra* ranged from 60% to 63% for the Dunn procedure and from 67% to 77% for the Newman-Keuls procedure. The modified Rogers distance for these species comparisons ranged from 0.87 to 0.92 (Kettler 1985; M. K. Kettler and G. S. Whitt, unpublished data). It would be premature to estimate absolute time limits for the effectiveness of this gene expression-distance approach until a variety of taxa has been examined.

For the Umbridae, the trees derived from gene-expressional data are congruent with the trees derived from structural gene data (genetic distance) and with some of the trees derived from morphological data. Because the species investigated include only a pair of closely related species and a pair of much more distantly related species, it is possible that in this analysis we have overlooked discontinuities in the rate of change in gene expression. The Poisson analysis was used in an effort to reveal possible discontinuities in rates of gene-expressional change rather than observed gene-expressional differences.

The fewest number of estimated gene-expressional changes (per tissue-enzyme) occurred between the congeners *U. limi* and *U. pygmaea* (0.31). The next fewer number of changes occurred between *Dallia* and *Novumbra* (1.08). The larger number of estimated gene-expressional changes between *Dallia* and *Novumbra* agrees with genetic-distance data (Kettler and Whitt 1984; Kettler 1985; M. K. Kettler and G. S. Whitt, unpublished data). Genetic distances indicate that although *Dallia* and *Novumbra* are more closely related to each other than to any other member of the family Umbridae, they probably diverged quite some time ago (fossil evidence suggests a possibly pre-Miocene divergence [Cavender 1969]). Members of the genus *Umbra* have also been identified from the Oligocene (Nelson 1972), and the number of gene-expressional changes calculated for *U. limi* and *U. pygmaea* versus *Dallia* are 1.10 and 1.17, respectively. (Note that over the same time period *U. pygmaea* has had more expressional changes than has *U. limi*.) Since the genetic-distance data suggest that the ancestor of the *Umbra* species separated from the *Dallia-Novumbra* lineage, a slightly greater but similar number of gene-expressional differences might be expected for the *Umbra* versus *Dallia-Novumbra* comparisons. However, more gene-expressional changes have occurred between *Novumbra* and *Umbra* (1.40 and 1.48, respectively) than between *Dallia* and the *Umbra* species (1.10 and 1.17, respectively). This difference might be explained by the reduced population size and restricted range of *Novumbra* (and its consequent effect on gene fixation) or by some other mechanism that would account for a more rapid accumulation of gene-expressional differences than structural-gene differences, such as selection (Kettler and Whitt 1986). Alternatively, *Novumbra* may actually be more distantly related to the rest of the umbrids than has previously been thought. Genetic-distance data suggest that *Novumbra* may be as closely related to a member of the family Esocidae (*Esox americanus*) as it is to *Dallia*. Perhaps *Novumbra* split off from the rest of the mudminnows even earlier than did *Dallia*, i.e., nearer the
origin of the family itself (from an unbrid-esocid ancestor). Since Esox species are known from the Paleocene (Wilson 1980, 1984), this split may have occurred as long ago as 63 Myr, in which case the greater number of gene-expressional changes would be expected for Novumbra. However, the problem still remains as to how, since they last shared a common ancestor (1.08/2 = 0.54 “changes per tissue-enzyme ago”), Novumbra has accumulated more gene-expressional changes than has Dallia. The relationship of Novumbra to the family Esocidae needs to be clarified before this problem can be properly addressed. A similar type of “problem” exists for U. pygmaea, which has had more expressional changes than U. limi over a similar time period. Although the extent of rate differences between U. limi and U. pygmaea is not of the same magnitude as that of the rate differences between Dallia and Novumbra, a similar non-time-dependent phenomenon appears to be involved.

Many more discontinuities of rate might have been observed if a more speciose family, with a broader spectrum of divergence times and/or habitats, had been chosen. If species of similar evolutionary age and species occupying a series of diverse habitats were examined, perhaps selective forces would become more apparent. Since the unbrid species that we have chosen differed more in relative divergence times than in habitat, we probably measured changes in gene expression that occur in a more time-dependent manner than do those that may occur via selection. Nevertheless, the accumulation of these gene-expressional differences, when considered over many tissues and enzyme loci, is sufficiently time related that we can construct trees with a branching pattern similar to the one based on enzyme structural differences (genetic distances), which in turn are believed to accumulate in a reasonably time-dependent manner. Because tissue-enzyme expressional differences are reflections of differences in tissue patterns of gene expression, these characters can help provide insights into the tempo and mode of evolution of regulatory genes controlling the steady-state levels of enzyme molecules. Thus, this approach provides a partial measure of gene regulatory divergence operating at one organismic level and another means of investigating the selective and stochastic forces underlying the evolution of different organismic physiologies.

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