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Evolution of the Differential Regulation of Duplicate Genes After Polyploidization

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Summary. In the 50 million years since the polyploidization event that gave rise to the catostomid family of fishes the duplicate genes encoding isozymes have undergone different fates. Ample opportunity has been available for regulatory evolution of these duplicate genes. Approximately half the duplicate genes have lost their expressions during this time. Of the duplicate genes remaining, the majority have diverged to different extents in their expression within and among adult tissues. The pattern of divergence of duplicate gene expression is consistent with the accumulation of mutations at regulatory genes. The absence of a correlation of extent of divergence of gene expression with the level of genetic variability for isozymes at these loci is consistent with the view that the rates of regulatory gene and structural gene evolution are uncoupled. The magnitude of divergence of duplicate gene expressions varies among tissues, enzymes, and species. Little correlation was found with the extent of divergence of duplicate gene expression within a species and its degree of morphological "conservatism", although species pairs which are increasingly taxonomically distant are less likely to share specific patterns of differential gene expression. Probable phylogenetic times of origin of several patterns of differential gene expression have been proposed. Some patterns of differential gene expression have evolved in recent evolutionary times and are specific to one or a few species, whereas at least one pattern of differential gene expression is present in nearly all species and probably arose soon after the polyploidization event. Multilocus isozymes, formed by polyploidization, provide a useful model system for studying the forces responsible for the maintenance of duplicate genes and the evolution of these once identical genes to new spatially and temporally specific patterns of regulation.

Key words: Isozymes – Gene duplication and regulation – Molecular and regulatory evolution – Polyploidy Fish – Teleosts

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Introduction

During the course of evolution there has been a general increase in the cellular DNA content of more complex forms of life (Britten and Davidson, 1971; Sparrow and Nauman, 1976). In some groups this increase in DNA has been accompanied by an increase in the number of multiple locus isozymes (Ohno, 1970). An increase in evolutionary complexity is reflected in the increased numbers of differentiated cell types and unique gene products within each cell type (Markert and Urspring, 1971). For example, Hopkinson et al. (1976) have estimated that approximately one-third of human enzyme loci exist in multiple forms. The origin and diversification of one multilocus system, hemoglobin, has been thoroughly documented (Ingram, 1961; Zuckerkandl, 1965; Perutz et al., 1965; Goodman et al., 1975). In the lower vertebrates, the phylogeny of creatine kinase (Eppenberger et al., 1971; Watts, 1975; Fisher and Whitt, 1978), lactate dehydrogenase (Whitt et al., 1973; Markert et al., 1975; Zuckerkandl, 1978) as well as other multilocus isozymes (Fisher et al., 1979) reveal a marked proliferation of isozyme loci which has been accompanied by an increasing restriction of their expressions to various differentiated tissues. The evolution of duplicate genes has also been studied in plants (Gottlieb, 1976; Garcia-Olmedo et al., 1977; Hart and Langston, 1977). The divergence in kinetic properties of isozymes encoded in duplicate genes has been demonstrated by Gottlieb (1977), and temporal divergence of multilocus isozyme expression has been demonstrated by Scandalios (1975).

How do the genes responsible for the different repertoires of enzymes and multilocus isozymes become differentially activated and modulated in the many diverse cell types? This question, which is one of the central ones in developmental biology, can be restated from an evolutionary perspective. How do these homologous genes, which were initially identical in their structure and regulation, come under separate developmental control and exhibit temporally and spatially specific patterns of gene expression. The differential expression of isozyme loci in various cell types or at different times of development is primarily the result of the evolution of regulator genes controlling the expression of the isozyme structural genes. The evolutionary events leading to the differential expression of originally identical genes are therefore of considerable interest to evolutionists and developmentalists.

Polyploid organisms provide an excellent opportunity to study the different evolutionary fates of duplicate genes. Ohno (1970) hypothesized that one or more rounds of polyploidy have been important in the early formation of the chordates (approximately 500 million years ago) and explain, in part, the great diversity of related isozymes present in the vertebrates today. Examination of more recent polyploids (50-100 million years ago) in a few fish orders has revealed that these polyploids express a significantly higher number of multiple locus enzyme systems than their diploid relatives (Allendorf et al., 1975; Engel et al., 1975; Ferris and Whitt, 1977a,b,c). The catostomid fishes of North America represent an entire family derived from a tetraploidization event 50 million years ago (Uyeno and Smith, 1972). The catostomid chromosome number and DNA content are approximately twice those of related diploid cypriniform taxa (Uyeno and Smith, 1972). In the 50 million years since the tetraploidization event (probably allopolyploid) giving rise to this family, and the subsequent transition to disomy, there has been ample opportunity for structural and regulatory gene evolution and differentiation (Ferris and Whitt, 1978a; Ferris et al. 1979).

The average catostomid has lost the expression of 53% of its duplicate gene copies through null mutations. The levels of duplicate gene expression retained are comparable to those in other tetraploid fish of approximately the same age (Allendorf et al., 1975; Engel et al., 1975; Ferris and Whitt, 1977c; Bailey et al., 1978).

The present study will focus on the evolution of the regulation of the expression of the duplicate genes which have been retained in the catostomids. The expression of a gene is defined as the outcome of all the genetic and epigenetic events leading to the production of the fully functional enzyme, whose activity is observed on a gel. We have found substantial differential expression of duplicate genes within and among tissues after 50 million years. The extent of divergence of expression of duplicate genes has been analyzed according to enzyme, tissue, and species. The degree of divergence of expression of duplicate gene copies has been compared with a number of parameters, including the taxonomic rank of a species, levels of genetic variability, physical and functional properties of the enzymes, as well as developmental and physiological properties of the tissues in which these duplicate genes are expressed. Lastly, we have been able to trace the probable phylogenetic point of origin of specific patterns of differential gene expression, for several multilocus isozyme systems.

Materials and Methods

The rationale for establishing isozyme homologies among species and the criteria and assumptions used in determining the number of genes expressed are given in Ferris and Whitt (1978a); and Ferris et al. (1979).

Adult fish were stored at -10°C and analyzed within two months. No differences in the isozyme patterns were detected between frozen and unfrozen specimens. However, one species, *Chasmistes brevirostris*, had been stored two years. Therefore, its patterns of gene expression should be regarded as preliminary. Fish were collected during the summer in post-reproductive stages in order to minimize seasonal and physiological fluctuations which might affect gene expression. In a previous survey of the catostomids, (Ferris and Whitt, 1978a) the duplicate locus expression of each enzyme was scored from one or two tissues in 15-30 individuals of a species. The ratios of isozymes, encoded by duplicate genes expressed in a given tissue exhibited low intraspecific variation. For the present study, the relative duplicate gene expression was determined for each of 10 tissues from an individual from each of 15 species. The low intraspecific variation was quantified on the basis of our examination of duplicate gene expression in each of 4 tissues from each of 11 individuals of *Carpiodes cyprinus*.

Extracts of tissues were prepared by homogenizing each tissue in two volumes of 0.1 M Tris HCl pH 7.0 at 4°C with a motorized Potter Elvehjem homogenizer. The enzyme extract was centrifuged at 27,000 x g at 4°C for 20 min. Approximately 50 µl of each supernatant was loaded into each of 10 slots in starch gels (Buchler, Inc., New Jersey) and after vertical electrophoresis were stained histochemically at 37°C and in the appropriate pH buffer for each enzyme according to procedures in Shaw and Prasad (1970)[1]. A more detailed discussion of materials and methods is provided in Ferris and Whitt (1978a).

[1]Enzyme abbreviations are: acid phosphatase (ACP), adenylate kinase (AK), aldolase (ALD), creatine kinase (CK), glucose phosphate isomerase (GPI), glycerol-3-phosphate dehydrogenase (G-3-PDH), lactate dehydrogenase (LDH), mitochondrial malate dehydrogenase (M-MDH), cytosol malate dehydrogenase (MDH), 6-phosphogluconate dehydrogenase (6PGD), and superoxide dismutase (SOD)

Care was taken to stop the staining process early so that the bands on the gel did not overstain. However, occasionally when one or more of the isozymes was present in disproportionately higher amounts (and activity) than the other isozymes, it was difficult to detect the isozymes present in minor amounts and then to stop the staining process before the rate of dye deposition became nonlinear for the isozymes in higher levels. The reaching of a plateau of staining intensity for the more active isozyme while the staining intensity change is still proceeding linearly for the less active isozyme would introduce a bias in our results in a more conservative direction. That is, the intensities of isozyme bands would appear to be more similar to each other than their actual activities (and number of molecules present) really were. Another source of error can be responsible for a non-stoichiometry of amount of dye deposited and amount of isozyme present. Enzymes present in higher concentrations in gels tend to be less catalytically efficient on a per enzyme molecule basis than the more dilute isozymes (Markert and Masui, 1969; Klebe, 1975). This bias also leads to a conservative error, i.e., there appears to be less divergence in the ratio of activity of duplicate gene expression than there really is. These sources of error were minimized when we employed the Klebe (1975) serial dilution procedure to determine the visual endpoints for quantitation of relative isozyme activities.

The relative contributions to isozyme activities of the two duplicate genes expressed within a tissue were determined in two ways. In the first case the relative intensities of the isozyme bands were determined for many enzymes by the serial dilution method of Klebe (1975). We examined duplicate gene expression in 90 tissues in this way (about 10% of all tissues examined) among the species and enzymes. This procedure used serial one-half dilutions of extracts (containing isozymes encoded by duplicate genes) loaded sequentially in the 12 slots of a starch gel. The gel was then histochemically stained after electrophoresis. The staining reaction was stopped before isozyme activity was detected in the channel receiving the extract in lowest dilution. The last dilution at which a band was visible was noted for each homopolymeric isozyme of the duplicate genes. The relative ratio of activities of these homopolymer bands was calculated, and fell within a series: 1:1, 1:2, 1:4, etc.

The second method for determining the relative contributions of the duplicate loci was direct visual estimation of the ratios without a serial dilution. These visual estimations were based on comparisons of unknown ratios with the ratios which had been elucidated by Klebe's procedure and on standards which we established by mixing different amounts of isozymes with known activity to give known ratios. As was the case for the serial dilution procedure the ratios which could be distinguished with a high degree of confidence differed by multiples of two, i.e., 1:1, 1:2, 1:4, etc.

In the case of some enzymes only one gene product was detected in one of the tissues whereas two gene products were present in one or more of the other tissues. One might interpret the presence of only one gene product in a tissue as the non-expression of one of the genes, or one might presume that both genes are expressed but that the level of one of the gene products is too low to be detected by histochemical staining procedures. Since we were unable to distinguish between these alternatives we chose to take the more conservative stance that the second gene product was present but in undetectably small amounts. Therefore we established a probable lower threshold level for different homopolymeric isozymes. These threshold limits for detecting homopolymers were estimated from experience with Klebe's serial dilution procedure. In instances

where only one homopolymeric band was observed, and no heteropolymers were observed, the other locus homopolymer was assumed to be undetectable, but present in a ratio of 1:32 for monomers, and 1:256 for dimers and tetramers. Such an assumption that the isozyme is present but just below the threshold of detectability again introduces a conservative bias into the results.

The ratios of subunit activities were in most instances able to be established from the ratios of the activities of the two homopolymeric isozymes. In some instances only one homopolymer was detectable and the other homopolymer was not. In these cases, the presence of heteropolymers demonstrated that the second locus was active, although at a very low level. From the ratios of homopolymer to heteropolymer activities on serial dilution gels it was possible to calculate the relative contributions of the subunit types, and the theoretical levels of the second, undetected homopolymer. Our data from the Klebe dilution experiments were generally consistent with the assumption that the different subunit types were assembling randomly and that the distribution of the homopolymeric and heteropolymeric isozymes was a binomial one. The genetic and molecular basis of one exception, the creatine kinase-A subunits, is described elsewhere (Ferris and Whitt, 1978b). The only other non-binomial isozyme pattern was observed for duplicate Ldh-A loci and is described in the results.

A necessary assumption that we have made is that the ratios of the isozyme staining intensities reflect the molar ratios of the different subunit types and these in turn reflect the relative expressions of the duplicate genes. This assumption does not appear unreasonable in light of Lewis and Gibson's (1978) recent report that differences in total ADH activity among strains of *Drosophila* was usually due to differences in the numbers of enzyme molecules, not to differences in their catalytic efficiency. However, the less likely alternative has not been excluded for the catostomids. The molar ratios of the subunits in different tissues (and therefore the ratios of the duplicate gene expression) were estimated by taking the square root of the ratio of homopolymer activities for dimers, and the fourth root of the ratio for tetramers. No adjustments were necessary for monomers. Because the ratios fall within a geometric progression, all statistical analyses were made on the $\log_{10}$ transformation of the ratios. The sign of the ratio was made positive when the more anodal isozyme predominated and negative when the less anodal isozyme predominated. In the case of all statistical calculations the absolute value was taken, leading to a more conservative estimation of the extent of divergence. For further comparisons using the mean level of divergence, the inverse log was taken of the mean.

Results

The patterns of duplicate gene expression, as revealed for all species, enzymes, and tissues exist in a continuum from nondivergent to highly divergent. The results will be presented in five sections, the first a primarily descriptive assessment of the various patterns of divergence of duplicate gene expression among tissues, the second an account of intraspecific variation in ratios of isozyme expressions, the third an analysis of the distribution of ratios of duplicate gene expression among tissues, the fourth a quantitation of the extent of divergence of duplicate gene expression among species, enzymes and tissues, and lastly, a phylogenetic analysis of the origins and distributions of specific tissue patterns of differential gene expression.

Patterns of Duplicate Gene Expression

Three categories have been established for the types of gene expression, in a given species and for a given enzyme. These categories are designated nondivergent, unidirectionally divergent, and bidirectionally divergent. In the nondivergent category, duplicate genes are equally expressed in all tissues in which the enzyme is expressed. In the unidirectionally divergent category, which includes most of the divergent patterns observed, the duplicate genes are expressed unequally in one or more tissues and the departure from the 1:1 ratio of activity is the consequence of either the more anodally or less anodally migrating isozyme predominating in all tissues in which such a departure occurs. The patterns of duplicate gene expression in the bidirectionally divergent category are also characterized by differential gene expression, but in this category there is no consistent predominance of one locus product over another among the various tissues. For example, one homopolymeric isozyme may be predominantly expressed in some tissues, and the other homopolymeric isozyme be predominant in its expression in other tissues. In the case of either unidirectional or bidirectional divergence, some duplicate genes can be equivalently expressed in some of the tissues. Some of the possible molecular and genetic bases for the categories of phenotypes will be discussed later.

An illustration of nondivergent expression in all tissues is shown in Fig. 1 for adenylate kinase (AK), in *Ictiobus bubalus*.[2] Nondivergent expression both within and among tissues is assumed to represent the ancestral pattern of duplicate gene expression since these duplicate genes were initially identical or nearly identical, and thus equivalently expressed.

The extent of differential locus expression among tissues in the unidirectionally divergent category can range from equivalent gene expression in most tissues, to nonequal expression in all the tissues in which the enzyme is expressed. An example of a subtle divergence in duplicate gene expression in seen for cytosol malate dehydrogenase (MDH-A) loci of *Cycleptus elongatus* in Fig. 2. Equal ratios of the homodimer activity are present in all tissues except gonad, where the A^2 locus expression predominates over that of the A^1 locus. A greater divergence of gene expression is seen for the mitochondrial malate dehydrogenase loci of *Hypentelium nigricans* in Fig. 3. In this species, 8 of 10 tissues have divergent ratios, with only brain and gill having a 1:1 ratio of activity. An examination of the staining intensities of the homodimeric bands, A^1_2 and A^2_2, reveals that among the 8 divergent tissues, heart, eye, spleen, and kidney are only slightly diverged from the 1:1 condition, whereas the duplicate genes are more differentially expressed in the other four tissues. In all tissues where divergent gene expression was detected, the A^1 gene predominated over the A^2 gene in expression. Another pattern of divergent gene expression is illustrated in Fig. 4. for *H. nigricans*. These patterns are more divergent than those shown before. The enzyme, glucosephosphate isomerase (GPI), is a dimer. The A and B loci, present in all diploid teleosts, appear to

[2]In the illustrations, loci related by duplication 50 million years ago are designated by numerical superscripts in order of the decreasing electrophoretic mobility of their isozymes. Only in a limited number of cases can the orthologous relationships between these recently duplicated genes be inferred from their relative electrophoretic mobilities (Ferris and Whitt, 1978a). Isozyme loci formed by duplication much earlier in chordate evolution are denoted with different capital letters. The orthologous relationships of these loci among species can be readily determined

Duplicate AK-A Locus Expression in Tissues of *Ictiobus bubalus*

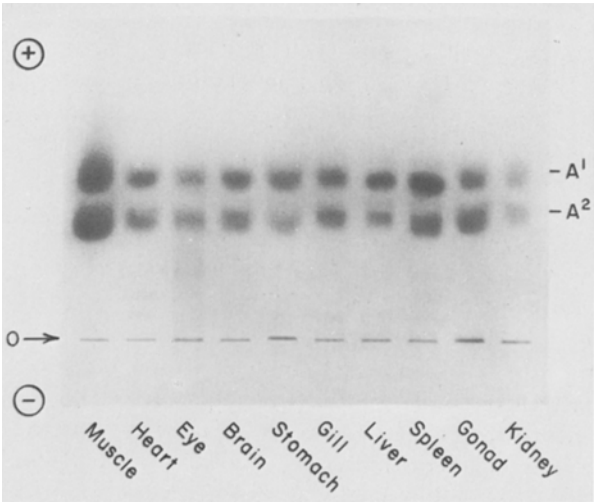


Fig. 1. Expressions of duplicate adenylate kinase loci in tissues of *Ictiobus bubalus*. The isozymes encoded by the duplicate loci are equivalently expressed in each of the tissues

MDH-A Gene Expression in Tissues of *Cycleptus elongatus*

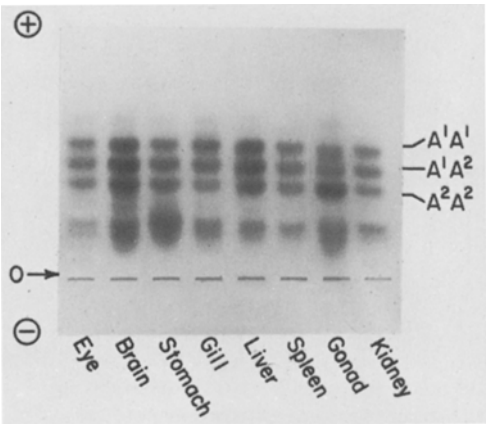
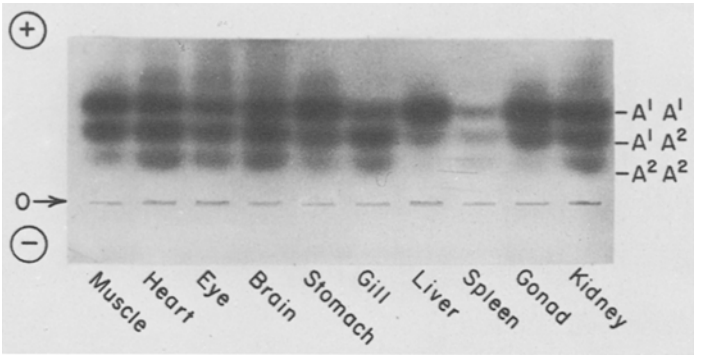


Fig. 2. Expression of duplicate cytosol malate dehydrogenase-A loci in tissues of *Cycleptus elongatus*. Equivalent gene expression is observed for most tissues, however, a slight divergence of gene expression is evident in one of the tissues, gonad

have arisen by an ancient polyploidization event over 500 million years ago (Avise and Kitto, 1973; Whitt et al., 1976; Fisher et al., 1979). The B locus is restricted in its expression to muscle, heart, and gonad, whereas duplicate A loci are expressed in all tissues except muscle. The homodimeric isozymes encoded by the recently duplicated A loci are present in unequal activities in all the tissues except kidney, where it is 1:1. In those nine tissues with divergent gene expressions, all nine exhibit a substantial asymmetry of duplicate gene expression.

An example of a species (*Cycleptus elongatus*) in which duplicate genes are differentially expressed in all tissues is shown in Fig. 5. The enzyme, lactate dehydrogenase (LDH), is a tetramer and is encoded by a basic set of three loci, A, B, and C in most diploid teleosts (Whitt et al., 1973; Markert et al., 1975). The A and B loci probably arose by duplications nearly 500 million years ago and the C locus probably arose in

Mitochondrial Malate Dehydrogenase Isozyme Patterns in Tissues of *Hypentelium nigricans*



Differential GPI-A Locus Expression in Adult Tissues of *Hypentelium nigricans*

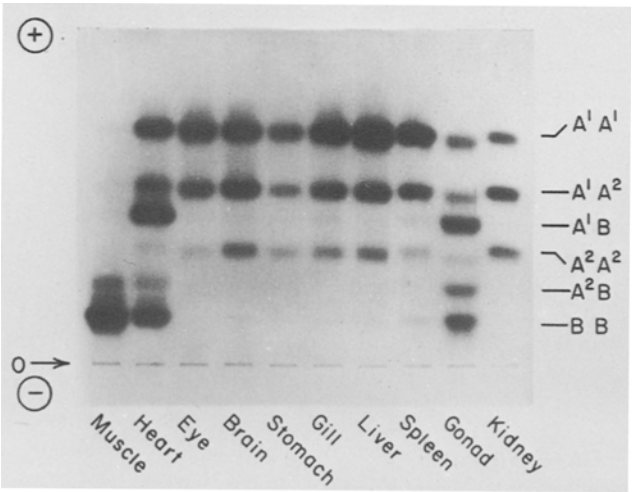


Fig. 3. Differential expression of duplicate mitochondrial malate dehydrogenase loci in tissues of *Hypentelium nigricans*. The duplicate loci are differentially expressed in most tissues of this species, and the pattern is of the unidirectional type. There are also some differences among tissues in the ratios of differential gene expression

Fig. 4. Differential expression of glucosephosphate isomerase-A loci in tissues of *Hypentelium nigricans*. Extensive divergence in the expression of duplicate Gpi-A loci have occurred within and among tissues. The Gpi-B locus is singly expressed in tissues of this species due to genic diploidization

the first bony fishes (Markert et al., 1975). All three loci (A, B, and C) were presumably duplicated during the tetraploidization of the catostomids and subsequently the duplicate of the C locus has undergone genic diploidization in this species, while the A and B loci have remained duplicated. Immunoprecipitation combined with starch gel electrophoresis was employed to determine which of the isozymes in Fig. 5 are encoded by B subunits. The homotetramers have been denoted in the figure. The B^2 homotetramer predominates over the B^1 homotetramer in all tissues. The duplicated

LDH Isozyme Patterns in Tissues of *Cycleptus elongatus*

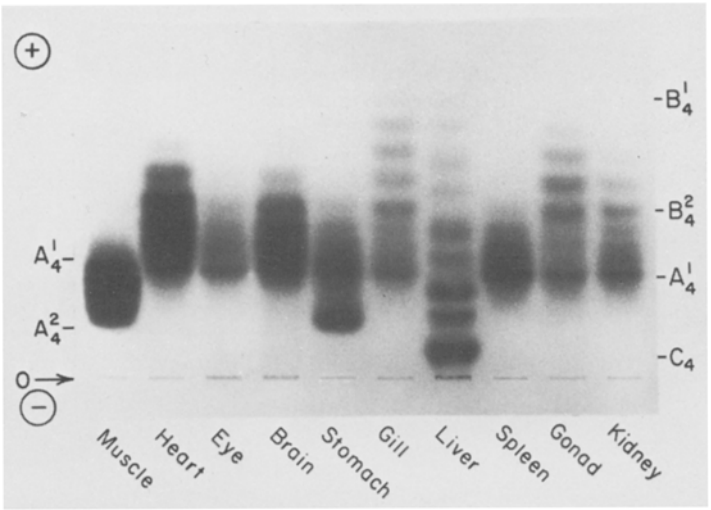


Fig. 5. Spatially specific expression of duplicate LDH loci in *Cycleptus elongatus*. The Ldh-C locus has functionally diploidized, but both Ldh-A and Ldh-B genes have been retained in duplicate. Both sets of duplicate loci show considerable differential expression within and among tissues

Differential Expression of Duplicate Creatine Kinase-B Loci in Tissues of *Carpiodes cyprinus*

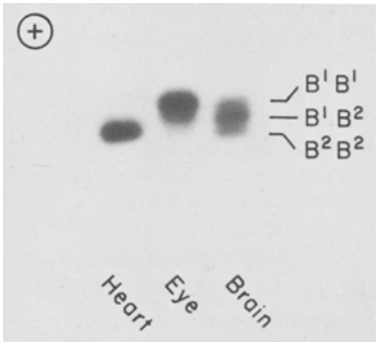
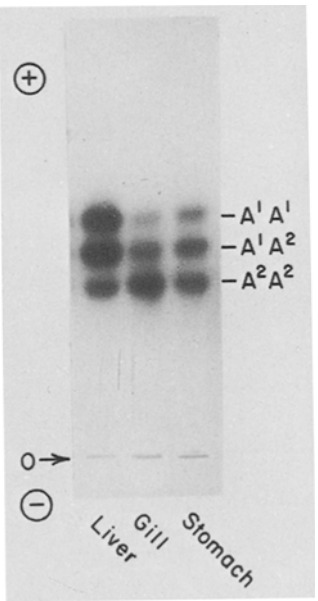


Fig. 6. Differential expression of duplicate Ck-B loci in tissues of *Carpiodes cyprinus*. The tissue differences in isozyme patterns in which the B_2^1 predominates in eye and the B_2^2 is exclusively expressed in heart is consistent with the differential regulation of these duplicate loci, and is referred to as a bidirectional pattern of divergence

A loci are expressed equally in muscle, whereas the A^1 homotetramer greatly predominates or is the only one expressed in all other tissues except stomach. However, in stomach there has been a reversal in the predominance of expression of the duplicate genes such that the A^2 predominates over the A^1. This "bidirectional divergence" appears to represent a more complex pattern.

One of the most striking patterns of differential gene expression is seen for the duplicate creatine kinase (CK) B loci in *Carpiodes cyprinus*, in Fig. 6. Both B loci are equivalently expressed in brain, and the dimeric isozymes are in a 1:2:1 ratio expected for random assembly of equal numbers of the two types of B subunits. These same duplicate genes are differentially expressed in other tissues. The B^1 homopolymeric isozyme predominates in eye tissue whereas in heart the B^2 isozyme is the only gene pro-

Differential Expression of Duplicate 6PGD Loci in Tissues of *Carpiodes velifer*



Differential LDH-A Gene Expression in *Cycleptus elongatus*

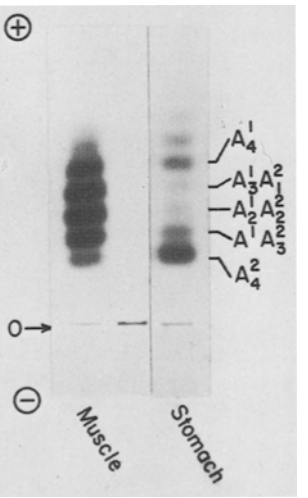


Fig. 7. Bidirectional pattern of divergence of duplicate 6-Pgd locus expression in three tissues of *Carpiodes velifer*. The A^1 locus expression predominates in liver, and the A^2 locus expression predominates in gill and stomach

Fig. 8. Duplicate Ldh-A locus expression in muscle and stomach of *Cycleptus elongatus*. The isozyme pattern of the skeletal muscle is a binomial one and consistent with free access and random assembly of both subunit types. Although the same LDH loci are approximately equally expressed in stomach the substantial reduction in the number of expected heteropolymers is consistent with the expressions of the two loci being spatially restricted to different cell types

duct detected. Another example of tissue differences in differential gene expression is illustrated in Fig. 7 for the dimeric 6-Phosphogluconate dehydrogenase in *C. velifer*. The A^1 locus expression predominates in liver, and the A^2 locus expression predominates in gill and to a lesser extent in stomach.

In most of the patterns of duplicate gene expression examined the ratios of homopolymers and heteropolymers are those expected for the random assembly of the subunits into all possible isozymes. These data suggest that although the duplicate genes can be differentially expressed within a tissue, the ratios of expression must be similar, if not identical, for the majority of different cell types making up that tissue. However, we have found a few non-binomial isozyme patterns which suggest that the duplicate loci are being preferentially expressed in different cell types. The isozyme distributions of each cell type would be binomial, but the different binomial patterns would add up to a non-binomial pattern for the whole tissue or organ. An extreme example of this spatially isolated gene expression would be for one duplicate locus to be expressed in one cell type and the other copy to be expressed in another cell type. Consequently, the two subunit types would have limited access to each other and thus no or only low

Differential LDH-A Gene Expression in the Digestive Tract of *Cycleptus elongatus*

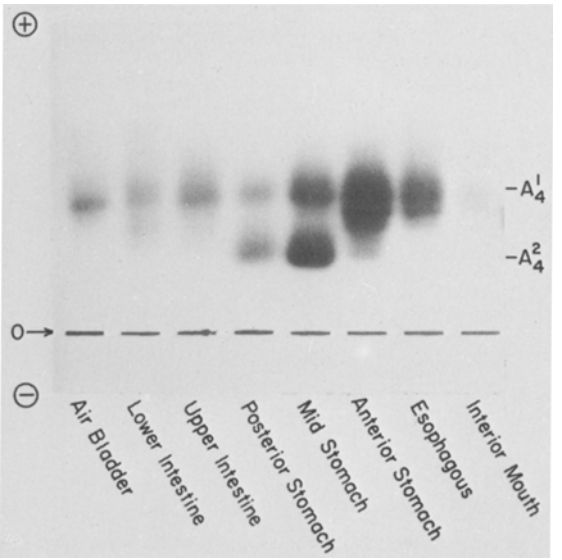


Fig. 9. Differential expressions of duplicate Ldh-A loci in different regions of the digestive tract of *Cycleptus elongatus*. The presence of heterotetramers in the anterior portion of the stomach and their absence in the middle portion of the stomach is consistent with a spatial isolation of A subunit synthesis

levels of heteropolymers would be detected in a tissue composed of a mixture of these cell types.

Examples of binomial and non-binomial LDH-A4 isozyme distributions are shown for two tissues of *Cycleptus elongatus* in Fig. 8. Near-binomial ratios of the five isozymes are present in muscle, yet in stomach all the heterotetramers are present in much lower levels than one would expect for the relative amounts of each of the two subunit types present. In order to determine whether the stomach isozyme pattern was an artifact of the homogenization procedure, equal amounts of the extracts of muscle and stomach were incubated 6 h at room temperature. There was no proteolytic digestion of heteropolymers in muscle by enzymes in stomach extract. However, we were unable to exclude the possibility that differential protease activity occurs *in vivo*. There was also no intrinsic instability of the heteropolymers in vitro when subjected to heat denaturation; a method which can sometimes provide insights into the stability of isozymes *in vivo* (Shaklee, 1975).

In order to gain a better understanding of the developmental and genetic basis of the non-binomial LDH isozyme pattern of the stomach, we followed the change in expression of the duplicate LDH-A genes in representative regions of the digestive tract. The results are shown in Fig. 9. The A^1 locus expression predominates over the A^2 locus expression in the esophagus and anterior stomach, and the $A_3^1A^2$ and $A_2^1A_2^2$ heteropolymers are present in these tissues in levels higher than that of the A_4^2 isozyme; a pattern more consistent with differential LDH gene function than differential lability of the heteropolymers. In the midstomach and posterior stomach, however, both

homotetramers are present but the heterotetramers are not. As one progresses along the upper and lower intestines, there is a progressive decline in the A_4^2 isozyme levels until only the A_4^1 isozyme is detected. The air bladder, a derivative of the esophagus, also has only A_4^1 activity.

Duplicate GPI-A Locus Expression in Brain of *Catostomus commersoni*

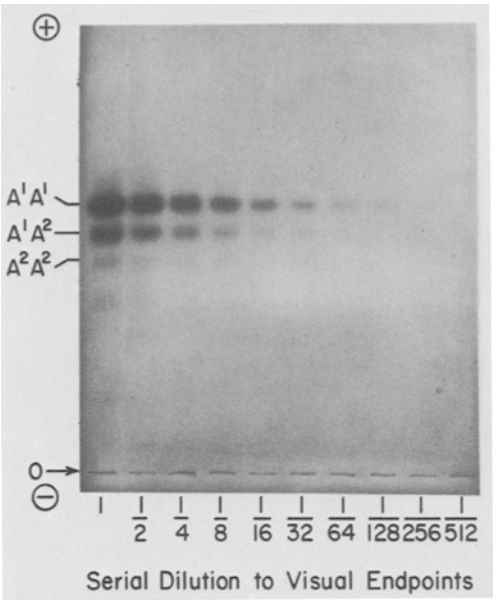


Fig. 10. An illustration of the serial dilution to visual end point method of Klebe (1975). The A_2^1 isozyme of *Catostomus commersoni* is last detected five serial 1/2 dilutions after the A_2^2 isozyme is last detected. Therefore there is approximately 32 times more activity in the A_2^1 homodimer than the A_2^2 homodimer

Variation in GPI-A Activity Within a Population of *Carpiodes cyprinus*

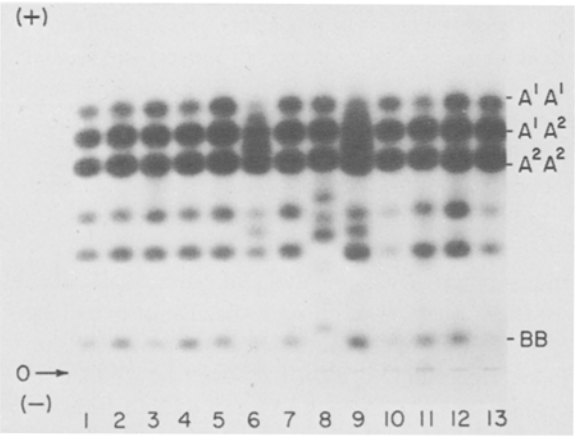


Fig. 11. The ratios of expression of duplicate Gpi-A loci in brain tissue of 13 individuals of *Carpiodes cyprinus*. Although there is some variation in the total GPI activity among the individuals, the relative ratio of activities of the A_2^1 and A_2^2 isozymes is approximately 1:4 in most individuals

Intraspecific Variation in Duplicate Gene Expression

A simple but powerful procedure has been developed recently by Klebe (1975) to quantitate the relative activity contributions of each of the isozymes, and thus by inference the ratios of the different subunits, encoded by the duplicate genes. An illustration of the application of Klebe's procedure is shown in Fig. 10. The homodimeric isozymes encoded in the duplicate GPI-A loci of *Catostomus commersoni* differ in the ratio of their activities by approximately a factor of five serial one-half dilutions. Therefore they are present in the approximate ratio of 32:1 and since the two subunits are assembling randomly, the ratio of the two subunit types contributed by all isozymes is $\sqrt{(32/1)}$, or 5.67:1. One repetition of the Klebe dilution procedure

Variation in CK-B Activity Within a Population of *Catostomus plebeius*

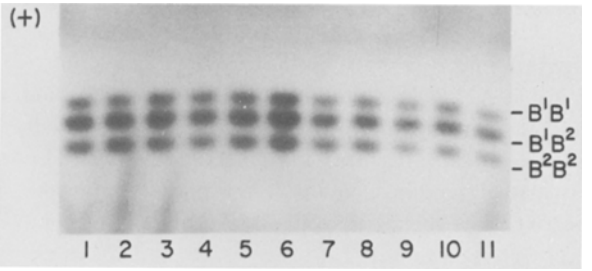


Fig. 12. The ratios of duplicate CK-B isozymes in brain tissue of 11 specimens of *Catostomus plebeius*. There is little detectable intraspecific variation from a 1:1 ratio of activity of the homopolymeric isozymes

Table 1. Intraspecific variation of duplicate gene expression in *Carpiodes cyprinus**

Enzyme	Tissue	Mean, log subunit activity ratio	Standard deviation, log subunit activity ratio
GPI-A	Brain	0.29	0.08
	Kidney	0.74	0.08
	Liver	0.52	0.18
MDH-B	Muscle	0.05	0.10
SOD	Liver	0.33	0.09

*Determined by 1/2 serial dilutions according to the method of Klebe (1975) with 11 specimens

for several tissues in several enzymes and species gave the same results with only slight variation (see appendix). An indication of the variance of the ratios of duplicate gene expression among individuals within a species is shown in Fig. 11. Extracts of brains from 13 individuals of *C. cyprinus* were electrophoresed to determine the relative expressions of duplicate GPI-A loci. Two of these individuals were heterozygous at one of the duplicate loci. Although *total* GPI activity varies somewhat from individual to individual, perhaps due to variation in the efficiencies of homogenization, the relative staining intensities of the different isozymes within each individual remain nearly the same, as presumably do the ratios of duplicate gene expression. In those cases where the ratios varied, as in slots 5 and 11, it was less than a factor of two. Since differences in the ratios of duplicate gene expression exist among species it is not surprising that some variation is expressed within a species. Another example of the low level of intraspecific variation in the relative expression of duplicate loci is shown for *Catostomus plebeius*. The CK-B loci are normally expressed equally in brain. The CK-B isozyme patterns of eleven individuals shown in Fig. 12 are in an approximate 1:1 ratio with little detectable variation.

To quantitate the amount of variation in the ratios of duplicate gene expression, the ratios of duplicate gene expression in each of 4 tissues in 11 individuals of *Carpiodes cyprinus* were determined for 3 enzymes. The measurements were made with the Klebe technique, using serial 1/2 dilutions. The results are shown in Table 1, and indicate that the standard deviations are rather small, and in accord with our previous observations of low levels of intraspecific variation of the ratios of duplicate gene expression. The low level of variable expression is assumed to hold for other tissues and enzymes, although it is not unreasonable to expect that some enzymes in some species will show variation in the regulation of duplicate gene expression.

The Distribution of Ratios of Duplicate Gene Expression Among Tissues

The ratios of activities of the isozymes encoded in duplicate loci among the different tissues of 15 species are given in the Appendix. Usually the same 10 tissues were analyzed in each species except *Xyrauchen texanus,* where spleen was omitted because it was too small to recover sufficient enzyme activity. A total of 15 different enzymes (15 pairs of duplicate loci) were studied. However, different numbers and combinations of these duplicate locus sets were examined within each of the species. An average of 8 enzymes (8 duplicate locus sets) was studied for each species. Usually less than the maximum possible 15 enzymes were able to be examined for each species because duplicate gene copies have been lost for different enzymes in different species. Both the original ratios of gene activities and the log transformations of these ratios are given. A (−) prefix in the log values indicates that the less anodally migrating homopolymeric isozyme predominated in expression over the more anodal isozyme. Blanks in the "cells" indicate that no isozyme activity was detected. The average of the ratios for each tissue and each enzyme are also shown for the 15 species.

The ratios of subunits encoded in duplicate genes were examined for a total of 864 tissues among the 15 species. The distribution of these ratios is given in Table 2, and their frequencies illustrated in Fig. 13. Nondivergent expression only constitutes 41% of all observations but is the most frequently encountered outcome compared to any

Table 2. Distribution of activity ratios of subunits encoded in duplicate genes in all tissues for 15 enzymes and 15 species

Ratios of Duplicate Gene Expression	Log (ratio)	Number of Duplicate Locus Sets	Subunit Number*
1:1.00	0.00000	356	D, M, T
1:1.19	0.0753	6	T
1:1.41	0.1505	112	D, T
1:1.68	0.2258	8	T
1:2.00	0.3010	115	D, M, T
1:2.38	0.3763	3	T
1:2.83	0.4515	84	D, T
1:3.36	0.5268	11	T
1:4.00	0.6020	80	D, T, M
1:5.66	0.7526	41	D
1:8.00	0.9031	17	D, M
1:11.31	1.0536	3	D
1:16.00	1.2041	27	D, T
1:32.00	1.5052	1	M
	Total	864	

*M = monomers, D = dimers, T = tetramers. The subunit types for a specific ratio are listed in order of decreasing number of observations. Mean ratio of divergence for all classes = 1:1.85. Mean ratio of divergence for divergent classes only = 1:2.83

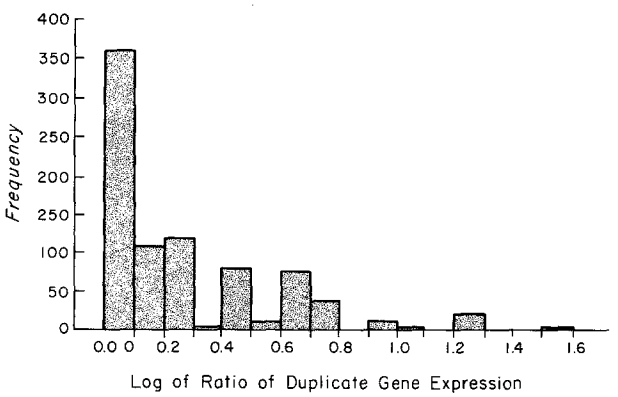


Fig. 13. The number of duplicate gene sets of each of the series of given ratios. These data are obtained for duplicate gene expressions in a total of 864 tissues examined for 15 species and 15 enzymes

Table 3. The distribution of nondivergent (A), unidirectionally divergent (B), and bidirectionally divergent (C), categories of duplicate locus expression in tissues of each Catostomid species*

Species	Number of Duplicate Sets	Categories A Number	A %	B Number	B %	C Number	C %
C. elongatus	13	3	23	7	54	3	23
I. bubalus	11	7	64	3	27	1	9
C. cyprinus	10	2	20	6	60	2	20
E. sucetta	6	0	0	5	83	1	17
E. oblongus	8	0	0	5	63	3	37
E. tenuis	6	0	0	5	83	1	17
M. melanops	7	0	0	6	86	1	14
M. duquesnei	7	0	0	6	86	1	14
M. erythrurum	6	0	0	5	83	1	17
H. nigricans	7	0	0	5	71	2	29
C. commersoni	8	0	0	6	75	2	25
C. catostomus	9	2	22	5	56	2	22
C. discobolus	8	1	13	6	75	1	12
C. brevirostris	9	1	11	7	78	1	11
X. texanus	10	1	10	7	70	2	20
Totals	125	17	14%	84	67%	24	19%

*A category - all tissues show 1:1 ratio for a given enzyme
B category - 1 or more tissues show a departure from a 1:1 ratio, with the same isozyme predominating in each case
C category - 2 or more tissues showing a departure from a 1:1 ratio, with one isozyme predominating in some tissues, the other predominating in other tissues

specific divergent ratio. The majority of tissues, 59%, show some degree of divergence of duplicate gene expression within them.

The relative frequencies of the three categories of divergence were determined for the 15 species. These data are shown in Table 3. Nondivergent expression among all tissues is present in 14% of the sets of patterns, unidirectionally divergent 67%, and bidirectionally divergent, 19%.

The extent of divergence of duplicate gene expression among tissues was then examined in more detail within the unidirectional category. For a given enzyme, the fraction of tissues was determined which showed nonequivalent expression of duplicate genes in an individual; e.g., 1/9 of the tissues show divergent expression in Fig. 2 for

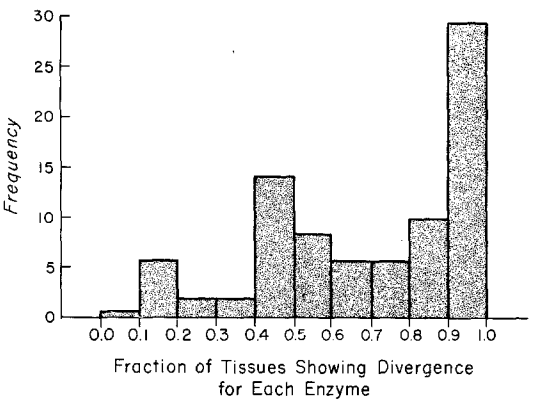


Fig. 14. The extent of divergence of duplicate gene expression among tissues in the unidirectionally divergent category. The horizontal axis represents the fraction of tissues in which the duplicate genes were differentially expressed for all the enzymes in the 15 species of catostomids. The vertical axis indicates the number of times a particular fraction of tissues exhibited a pattern of differential gene expression. See the text for a discussion of the relative contributions of monomeric, dimeric, and tetrameric enzymes in the peaks

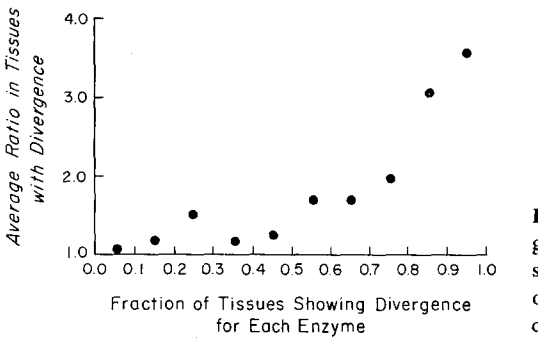


Fig. 15. The average ratio of duplicate gene activities in each tissue for those tissues with divergent expression for each of the intervals (fraction of tissues with divergent expression) in Fig. 14

MDH-A, and 8/9 tissues for GPI-A in Fig. 4. The numbers were converted to decimal equivalents, and the frequency of "sets" showing increasing proportions of tissues with divergent ratios is shown in Fig. 14. Three peaks are seen, and these are largely accounted for by contributions of enzymes with different subunit numbers. The peak in the 0.11-0.20 range is due mostly to the monomer, AK, the peak in the 0.41-0.50 range to the various dimeric enzymes, and a third of the 0.81-1.0 peak to the tetramers lactate dehydrogenase and aldolase. As we shall show later, AK isozymes tend to be nondivergent, and LDH isozymes highly divergent.

Is there an association of the magnitude of divergence of duplicate gene expression within a tissue with the magnitude of divergence that one sees among tissues? Only those tissues were included which showed divergent ratios among all the enzymes and species. The average ratio of duplicate gene expresisons was determined for each of the tissues for each 0.1 interval in Fig. 14. These averages are plotted in Fig. 15. Generally a species exhibiting considerable divergence in gene expression among its tissues will for the same enzyme show high degrees of divergence of gene expression within tissues.

We also investigated whether the most anodal or less anodally migrating isozyme of each duplicate set was predominant in its expression in a given tissue. We found that in 320 tissues the more anodally migrating product predominated, and in 190 tissues the less anodally isozyme predominated, over all enzymes and species. The difference from

the expected equal distribution is highly significant with a chi-square test (df = 1, chi-square = 33.1, $p < 0.001$).

Divergence of Duplicate Gene Expression Among Species, Enzymes, and Tissues

As was mentioned earlier, 59% of the tissues over all species and enzymes have some degree of divergence of duplicate gene expression. From Table 2 it is calculated that the average ratio of divergence, when there is a commitment to divergence, is 1:2.83. The average extent of divergence of all duplicate gene expressions, including divergent and nondivergent, is 1:1.85. The extent of divergence of duplicate gene expression was then determined for the different species, enzymes and tissues. Both divergent and nondivergent ratios were included in the averages.

The average divergence of duplicate gene expression in all tissues for each species is given in Table 4. The species with least divergence was the morphologically primitive *Ictiobus bubalus* and the species with the most divergence was the relatively morphologically advanced *Catostomus discobolus*. There was little correspondence, however, with degree of divergence of gene expression and the taxonomic rank for the remaining species. One measure of taxonomic rank, or primitiveness, is the number of duplicate genes which have been retained (Ferris and Whitt, 1978a). A Wagner tree constructed from complete losses of duplicate gene expression reveals a general trend

Table 4. Average divergence of duplicate gene expression for all enzymes and tissues in 15 species of Catostomids

Species	% Duplicate Genes Expressed	Ratio of Divergence of Duplicate Genes
CYCLEPTINAE		
Cycleptus elongatus	65	2.03
ICTIOBINAE		
Ictiobus bubalus	60	1.18
Carpiodes cyprinus	55	2.02
CATOSTOMINAE		
Erimyzon sucetta	45	1.88
Erimyzon oblongus	45	1.70
Erimyzon tenuis	35	1.52
Minytrema melanops	35	1.64
Moxostoma duquesnei	35	1.99
Moxostoma erythrurum	35	1.55
Hypentelium nigricans	35	1.79
Catostomus commersoni	50	2.08
Catostomus catostomus	45	2.12
Catostomus discobolus	40	2.37
Chasmistes brevirostris	45	2.01
Xyrauchen texanus	50	1.80

Table 5. Extent of divergence with increasing taxonomic distance

Taxonomic Comparison	Number of Shared Tissues	Index of Divergence*
Between species		
Moxostoma duquesnei vs. *M. erythrurum*	49	1.47
Erimyzon sucetta vs. *E. oblongus*	40	1.52
Between genera		
M. erythrurum vs. *Hypentelium nigricans*	40	1.53
Ictiobus bubalus vs. *Carpiodes cyprinus*	44	1.63
Between tribes		
H. nigricans vs. *Catostomus discobolus*	48	2.01
M. duquesnei vs. *C. catostomus*	58	2.05
Between subfamilies		
Carpiodes cyprinus vs. *M. duquesnei*	40	2.06
Cycleptus elongatus vs. *Minytrema melanops*	32	2.74

*Computed by summing the differences in ratios between corresponding tissues and homologous enzymes, dividing by the number of tissues, and taking the inverse log of the mean

Table 6. Divergence of duplicate gene expression for different enzymes averaged over 15 species

Enzyme	Number of Tissues	Ratio of Duplicate Gene Expression	Average Heterozygosity, Hd*	% Genic Diploidization in 30 Species
CK-A	4	1.00	0	83
ACP	18	1.02	0	87
AK-A	105	1.24	0.033	20
SOD	143	1.46	0.043	0
6PGD	82	1.49	0.053	13
GPI-B	30	1.52	0.197	37
MDH-A	82	1.62	0.006	33
M-MDH	136	1.99	0.024	10
MDH-B	7	2.10	0.083	73
ALD-C	6	2.18	0	77
G3PDH	6	2.38	0.023	53
CK-B	38	2.40	0	0
LDH-B	88	2.59	0	33
LDH-A	10	2.73	0	97
GPI-A	109	3.35	.132	10

*Based on a somewhat larger number of species (19)

for morphologically primitive species to retain the most functional duplicates, and advanced species the least (Ferris and Whitt, 1978a). The percent gene duplication is also shown in Table 4. A regression of this variable against the ratio of divergence showed essentially no correlation ($r = 0.02$).

There does appear to be a trend toward the divergence of duplicate gene expression with taxonomic divergence when homologous enzymes are compared among species. For 8 species pairs we compared corresponding tissues for each enzyme, and calculated the difference in log (ratio) of divergence. These differences were summed over all enzymes in the species, divided by the total number of tissues compared and the inverse log taken. The mean ratio will be referred to as the "index of divergence", and these indices are shown in Table 5. Species within a genus have similar patterns of divergence. More distantly related species have greater differences in their enzyme electrophoretic mobilities and extent of divergence of their duplicate gene expression.

The average level of divergence of duplicate genes for each of the 15 enzymes is shown in Table 6. These enzymes have been ranked from the least divergent in duplicate gene expression, CK-A, to the most divergent, GPI-A. A comparison was made of the extent of divergence of expression for isozymes encoded in duplicate loci formed by ancient gene duplications prior to the origin of the teleosts (e.g., LDH-A, B; CK-A, B; MDH-A, B; and GPI-A, B). The extent of divergence between the recently duplicated genes (by tetraploidy in the catostomids) is sometimes surprisingly similar for both members of the set formed by the more ancient duplication. MDH-A and B duplicate locus sets have similar extents of divergence, as do LDH-A and B sets as shown by their close proximity in the list of Table 6. However, the duplicate GPI-A

Table 7. Extent of tissue restriction of the enzymes

Enzyme*	Fraction of Tissues in which the Enzyme is Expressed
CK-A	0.20
ACP	0.90
AK-A	0.96
Sod	0.96
6PGD	0.92
GPI-B	0.34
MDH-A	0.92
M-MDH	0.98
MDH-B	0.12
ALD-C	0.20
G3PDH	0.10
CK-B	0.26
LDH-B	0.81
LDH-A	1.00
GPI-A	0.92

*Ranked in order of increasing divergence of duplicate gene expression

and B and CK-A and B loci appear to behave relatively independently in their extent of divergence.

The question of whether divergence of duplicate gene expression is related to functional or structural properties of the enzymes encoded in the duplicate genes was also investigated. A regression of extent of divergence of an enzyme among the 15 species in Table 6 with the subunit molecular weights of the enzymes given in Hopkinson et al. (1976) showed essentially no correlation ($r = 0.10$).

An important property of an enzyme is the extent of polymorphism and heterozygosity of the gene(s) encoding it. We attempted to determine whether a relationship between the extent of divergence of expression of duplicate loci and the average heterozygosity of these loci existed. The average heterozygosity of the duplicate loci (Table 6) was determined from 19 species of catostomids (Ferris and Whitt, in preparation). Sample sizes were too small to generate heterozygosity estimates for enzymes in three species in this analysis. Therefore enzyme heterozygosity estimates were based on a somewhat larger sample of species for a more reliable indication of genetic variability. No correlation exists between levels of heterozygosity and extent of divergence of gene expression ($r = 0.12$).

Table 8. Divergence of duplicate gene expression among different tissues

Tissue	Number of Tissues Examined	Ratio	Log Ratio	Standard Deviation (log)	Stage of Appearance in Development*
Brain	104	1.47	0.17	0.26	14
Eye	103	1.72	0.24	0.28	15
Muscle	68	1.73	0.24	0.32	17
Gill	84	1.81	0.26	0.26	23
Gonad	84	1.83	0.26	0.29	--
Kidney	103	1.90	0.28	0.32	15
Heart	87	1.91	0.28	0.38	17
Stomach	78	1.96	0.29	0.30	--
Spleen	77	2.10	0.32	0.32	--
Liver	76	2.29	0.36	0.34	24

*From Long and Ballard (1976) for *Catostomus commersoni*

Some of the less divergent patterns of duplicate gene expressions encountered could indicate that these duplicate genes might be more susceptible to loss in the future. If increasing divergence of duplicate gene expression reflects a greater functional specialization, then there should be a negative correlation of this variable for an enzyme and the percent of species in which the same enzyme has already undergone genic diploidization. The percent of 30 species from Ferris and Whitt (1978a) which have lost duplicate gene expression is given for each enzyme in Table 6. We found little correlation of divergence of duplicate gene expression and extent of diploidization ($r = 0.13$). These results tend to exclude the hypothesis that significant functional specialization has evolved in this relatively early period after polyploidization. These results are consistent with the hypothesis that regulatory divergence may precede significant functional divergence of the isozymes.

A measure of an enzyme functional specialization is the extent to which it is expressed in a number of tissue types. Less specialized enzymes should generally be expressed in a greater array of tissue types. Both copies of recently duplicated genes tend to be expressed to some extent in the same tissues as the corresponding single locus in diploids. The enzymes were ranked from the least divergent to the most divergent as in Table 6 and as seen in Table 7, there was no strong correlation of extent of divergence of the duplicate gene expression with the number of tissues in which the enzyme activity predominates ($r = 0.31$).

It is well known that some enzymes tend to be restricted in their expression to a few tissues and some enzymes tend to be expressed in most tissues. In addition,

multilocus isozymes such as LDH-A_4 and LDH-B_4 can predominate in quite different tissues. It was proposed, as a working hypothesis, that the divergent expressions of the duplicate loci in catostomids represent an early stage in the evolution of the tissue specificity of isozyme expression. We analyzed 10 tissues among the 15 species for the average extent of divergence of duplicate genes within them. The results are shown in Table 8 and the tissues are ranked from least divergent duplicate gene expression (top) to the most divergent duplicate gene expression (bottom). Brain is the most conservative with respect to divergent ratios of gene expression (1:1.47) and liver is the most divergent (1:2.29). The standard deviation of each of the log ratios is given in the 2nd column from the right. In general, the standard deviations are similar to the means. Several t tests were performed to gain some understanding of the significance of the observed differences among tissues. As might be expected, the extremes, brain and liver, are highly significantly different in their average extents of divergence of duplicate genes ($t = 4.29$, $df = 178$, $p < 0.001$). The values for brain are also significantly different from those for heart but to a lesser extent ($t = 2.67$, $df = 205$, $p < 0.01$). Differences between brain and eye, however, are not significant ($t = 0.675$, $df = 205$, $p = 0.50$).

Some caution should be exercised in interpreting differences among the average ratios for the different tissues. An analysis of variance of the data for species, tissues, and enzymes revealed significant tissue x enzyme and species x enzyme interaction effects, although the tissue x species interaction effect was not significant. The more extreme values for those tissues in the ranking of Table 8 are still significantly different, but a more precise measure of the significance of the differences will have to await the acquisition of additional data.

Zuckerkandl (1968) has persuasively argued that the rate of functional and regulatory evolution, for genes expressed in the early embryo, would be expected to be slower than the rates for genes activated later in life. In the light of this proposal, it is instructive to compare the extent of divergence of duplicate gene expression in tissues and organs which appear early in development, with the extent of divergence exhibited by tissues which appear later in development. The ontogenetic sequence of the morphogenesis of these tissues is available in detail for one catastomid, *Catostomus commersoni* (Long and Ballard, 1976), and to a lesser extent for *Erimyzon sucetta* (Shaklee et al., 1974). The developmental stages at which these tissues are morphologically first recognizable for *C. commersoni* are given in Table 8. Interestingly, brain and muscle, which have low ratios of duplicate gene divergence within them, appear earlier than gill or liver, which have more divergent ratios. Stomach tissue, although not listed, also tends to appear later in development and coincides with the absorption of the yolk sac (Armstrong and Child, 1965). This tissue is also characterized by highly divergent duplicate gene expression. Since the duplicate genes we are studying arose a very long time after the ontogenetic sequence of the morphogenesis of the tissues was established, any developmental correlation with extent of differential duplicate gene expression would not be a case of "ontogeny recapitulating phylogeny". However, it may be that the evolution of the differential regulation of these relatively recently duplicated genes is proceeding more slowly in the tissues formed during early embryogenesis and that this slowness coincides with what might superficially appear to be a recapitulation (Zuckerkandl, 1968).

Table 9. A comparison of enzyme catabolism and levels of mitosis in different rat tissues

Tissue	Radioactive Index	Protein Turnover**
Skeletal muscle	0	3.27
Brain	0	4.13
Heart	0	4.65
Kidney	4.0	8.07
Spleen	--	10.81
Liver	0.8	11.40

*A measure of extent of mitosis, from Schultze and Oehlert (1960)
**Based on ^{3}H: ^{14}C ratio after 10 days, Table 1 in Dan and Masters (1976)

Perhaps the ability to evolve a divergent ratio of activity of duplicate genes among tissues is in part related to the degree of metabolic homeostasis of the tissues and the level of metabolism of the cells in these tissues. A summary of the results from studies on rats by two different laboratories is shown in Table 9. One study shows the degree of mitotic activity in rat tissues as measured by the uptake of labelled thymidine; the other study shows the rate of total protein turnover in a number of tissues from rats. The tissues studied were at a steady state so that the rates of synthesis are equal to the rates of degradation. Muscle, brain, and heart are characterized by essentially no cell division, and low synthesis and turnover of proteins. These are the same tissues with low levels of divergence of duplicate gene activities. In constrast, kidney, spleen, and liver show high mitotic activity, high protein synthesis and turnover as well (Table 8) as more divergent ratios of duplicate gene expression in catostomids. The possible contributions of differential synthesis and degradation of duplicate gene products to the isozyme patterns in the various tissues will be discussed later.

Probable Times of Appearance of Specific Patterns of Duplicate Gene Expression in Phylogeny

One advantage of studying patterns of differential gene expression among species of many genera is that one can investigate when these different patterns of gene expression arose, and the lability or stability of these patterns over time and repeated speciations. In much the same manner as determining the probable time of a loss of duplicate gene expression, the probable times of appearance of several patterns of duplicate gene expression in catostomid phylogeny were able to be determined. A phylogeny of catosetomids, based primarily on morphology, is given in Fig. 16. A discussion of the systematic relationships of the catostomids is given in Ferris and Whitt (1978a).

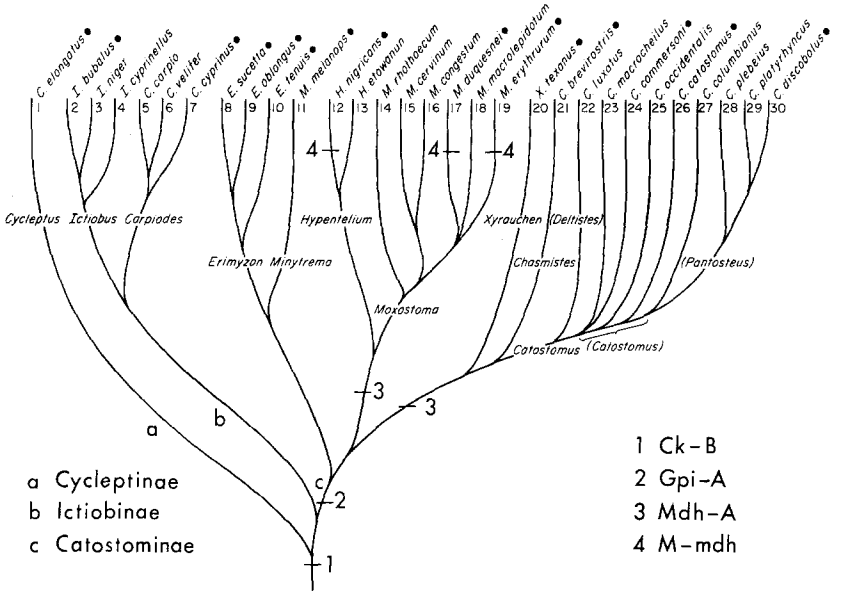


Fig. 16. Phylogeny of the catostomids based on morphological criteria. The 15 species included in the present analysis are indicated with a (•). The probable points of origin of specific patterns of differential tissue expression for four enzymes are indicated on this tree. The characteristic pattern of differential duplicate Ck-B locus expression probably arose shortly after the initial polyploidization; before the first speciation events. The establishment of the differential expression of GPI-A isozymes probably occurred early in the evolution of the Catostominae. Specific patterns of differential Mdh-A expression appeared independently in a number of lineages, and two of these are shown. M-mdh shows specific patterns in many instances, and as an example, three of these are shown in the genera of *Moxostoma* and *Hypentelium*

In the primitive species of *Cycleptus* and *Ictiobus* what may be presumed to be the ancestral condition of two CK-A genes has been retained, and these genes have not diverged in their expression among or within tissues. However, the CK-B genes have been retained in more genera and have undergone substantially more divergence in their expression than have CK-A genes. The distinctive pattern of differential expression of duplicate CK-B genes was shown in Fig. 6. This distinctive pattern appears in 8 of the 15 species. In fact, this characteristic pattern of duplicate gene expression is shared by species which are rather distantly related, e.g., *Carpiodes cyprinus* (Fig. 6) and *Catostomus discobolus* (Fig. 17). The pattern of differential CK-B gene expression (characterized by equal activity of the duplicate copies in brain, and the predominance of the B^1 locus in eye) is found in 14 of the 15 species and thus this pattern of gene regulation was probably established early in catostomid phylogeny. A slight predominance of the B^2 occurs in *E. oblongus*. Furthermore, 7 of the 15 species have no detectable CK-B gene expression in heart. This absence of CK-B activity in heart of some species suggests still another level of regulatory control of duplicate gene expression. Lack of any detectable CK-B gene expression in the heart of these 7 species probably represents a loss of function rather than the acquisition of CK-B activity in the heart

Creatine Kinase Isozymes of *Catostomus discobolus*

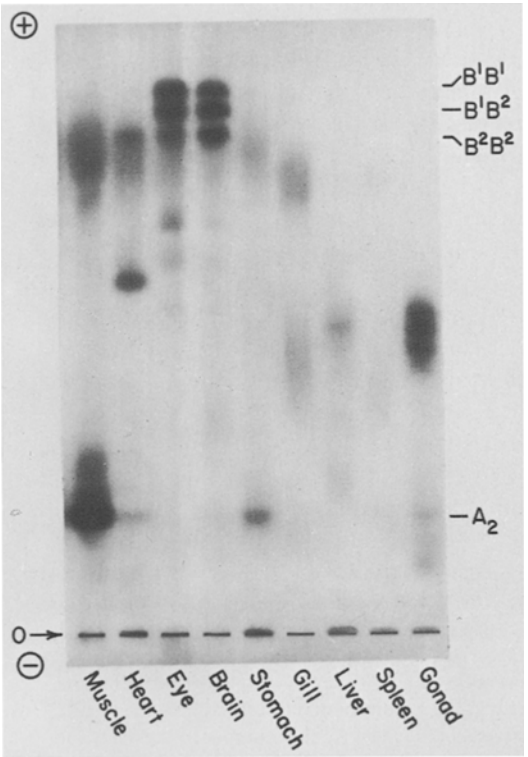


Fig. 17. Differential expression of duplicate Ck-B loci in *Catostomus discobolus*. The patterns of duplicate Ck-B locus expression in heart, eye, and brain of this species is similar to that pattern shown in the same three tissues of *Carpiodes cyprinus* in Fig. 6; a quite distantly related species

of the remaining 8 species since diploid cyprinids also have CK-B activity in heart (unpublished data).

The GPI-A tissue pattern of duplicate gene expression also arose early in phylogeny but probably later than the origin of the CK-B pattern. Two GPI-A loci are expressed in catostomids except the species in the genus *Erimyzon*. Two patterns of duplicate gene expression are found in the primitive catostomids. The first is equal expression of A^1 and A^2 in all tissues of *Ictiobus*, and the second is the predominance of A^2 locus expression in all tissues of *Carpiodes*. In the subfamily Catostominae the A^1 locus expression predominates over the expression of the A^2 locus. This pattern could have arisen as a structural mutation leading to reduced A^2 subunit activity in the line leading to the Catostominae. Alternatively, there could be lower levels of A^1 subunits than A^2 subunits in the species of the Catostominae.

A situation similar to that for GPI-A is found also for MDH-A. However, in the case of the latter isozyme system, different paths of divergence appeared even later, within the Catostominae. The line leading to the closely related genera *Moxostoma* and *Hypentelium* has the Mdh-A^2 locus predominating in its expression in many of the tissues, whereas the Mdh-A^1 locus predominates in its expression in the line leading to the closely related genera of *Catostomus*, *Chasmistes*, and *Xyrauchen*. Furthermore these two lines are distinguished by the relative expression of these duplicate loci

in the heart. In the first line the duplicate loci are equally expressed in the heart and in the second line unequally expressed.

Mitochondrial MDH patterns have evolved species-specific patterns in a number of species. Examination of the data in the Appendix for *Moxostoma erythrurum, M. duquesnei,* and *Hypentelium nigricans* reveals that these duplicate genes have diverged in their ratios of expression to varying extents in different tissues. The mitochondrial MDH loci are expressed in heart in a ratio of 1:2 in *M. duquesnei,* 1:1 in *M. erythrurum* and 1:1.41 in *H. nigricans*. These loci expressed in gill have a 1:1 ratio in *H. nigricans,* and divergent ratios in the other species. Other species in the Catostomidae also show different patterns of divergence of mitochondrial MDH expression among the tissues.

Lactate dehydrogenase-A remains duplicated in only one species, the primitive *C. elongatus*. As shown in Fig. 5, considerable divergence in the expression of the LDH-A loci occurred within and among tissues in this species over 50 million years. In the case of the related duplicate LDH-B, the commitment to diverge may have occurred rather early. All extant catostomids with duplicate LDH-B genes are highly divergent in the expression of these genes. It is difficult to discern any evolutionary patterns, and it is likely that many of the specific patterns arose independently and later in phylogeny in different taxa. One consistent feature in all the taxa is that the two B loci are always highly divergent in spleen, and frequently only one gene product was detectable.

The probable points of phylogenetic origin of four of the enzyme systems discussed above are shown in Fig. 16 for CK-B, GPI-A, MDH-A and mitochondrial MDH. The latter two enzymes have evolved more species-specific patterns than indicated among the species shown, and only a few patterns are included to illustrate the point that patterns of differential gene expression are probably continuing to be formed and to diverge.

Discussion

The catostomid fishes have lost the expression of half their duplicate genes in the 50 million years since their origin by polyploidization. We have found that for those duplicate genes which have been retained, their expression has diverged in 59% of the tissues of 15 species. Determination of the relative contribution of isozyme subunits encoded by each of the duplicate genes has allowed us to estimate the degrees of divergence of tissue expression of duplicate genes. When we studied the amount of divergence for all duplicate loci within tissues, for 15 enzymes and 15 species, we found that the average ratio of duplicate gene expressions was 1:1.8. The average ratios of gene expression for duplicate loci that have diverged was 1:2.8. The distribution of the gene expression, i.e., the number falling in the categories of 1:1, 1:2, 1:4, etc., in Table 2, is suggestive of a "decay" process from the nondivergent state, that is, there are progressively fewer and fewer instances of more highly divergent ratios of duplicate gene activity. For the remainder of this discussion, we will use the term "divergent ratio" to indicate a ratio different from 1 for the activity of subunits encoded by duplicate genes within a given tissue.

Two processes could be contributing to the divergence of the ratios. First, some of the divergent ratios could be brought about by structural gene changes altering catalytic efficiency or lability of one of the subunits. Lesions at structural genes may have

contributed to a small extent and will be discussed later. Second, and most important, is the accumulation of mutational differences at regulatory gene loci. The model for gene regulation in eukaryotes of Britten and Davidson (1969) specifies that each structural gene is regulated by a number of regulatory genes which are in a hierarchical relationship to one another. Wallace and Kass (1974) have also proposed multiple regulatory elements for each structural gene. A detailed proposal for how multiple regulatory genes can evolve developmental specificity has been provided by Zuckerkandl (1978). The duplication and subsequent divergence of these regulatory genes has been postulated to be a rich source of evolutionary novelty, at the level of control of gene expression (Ohno, 1970; Britten and Davidson, 1971) as well as morphological change (Ohno, 1970; Flickinger, 1975).

Chromosomal rearrangements have been postulated to bring about changes in gene regulation in some vertebrate groups (Wilson et al., 1974; King and Wilson, 1975). The regulatory changes observed for the catostomids have been accomplished in the absence of gross changes in chromosome structure and number, since most catostomids have 98-100 chromosomes (Uyeno and Smith, 1972).

The Number of Mutations Necessary for the Divergence of Gene Expression

Tetraploidy results in the duplication of the entire genome, which means that all regulatory genes are doubled as well as structural genes. In only 50 million years, the nucleotide sequences of some of the regulatory genes may not have accumulated sufficient differences to bring about divergence of regulation. Alternatively, sequence differences exist but for some enzyme loci they are "neutral" and there is no alteration of the ratio of duplicate gene expression in tissues. One might expect that over a period of 50 million years many regulatory genes will have accumulated at least a few nucleotide substitution differences which would result in differential transcription of a duplicate gene. In rare instances, as seen in Table 2 for the occasional highly divergent ratios, a single mutation affecting gene regulation could result in very divergent ratios, but in most instances such extreme divergence is probably the consequence of a series of mutations.

It is clear from our data that divergence within a given tissue can occur independently from divergence in another tissue. This observation taken together with the considerable heterogeneity observed among tissues in their divergence over many species, suggests that tissue divergence of duplicate gene expression may be a multiple hit process. Yet, we will shortly present data showing that the effects of these "hits" may be different in various tissue types. Each type of tissue is thought to have different concentrations of effector molecules with different affinities for different regulatory elements. Varying thresholds of effectors could be present in each tissue and determine the degree of transcription of a duplicate gene. The results we have obtained are in accord with the controller node stability model of Zuckerkandl (1978). In the case of some duplicate regulatory genes the accumulation of some nucleotide substitutions will lead to a complete loss of a duplicate gene expression in all tissues of an organism, or genic diploidization (Ferris and Whitt, 1978a).

It was particularly informative to compare the tissues in which an enzyme is expressed within a species. For each enzyme, these tissues constitute a "set" within the species, e.g., heart, eye and brain for CK-B in Fig. 6. For 15 species and enzymes,

there were 125 "sets" of tissues. Of these 125, 14% showed nondivergent expression of duplicate genes over all tissues for an enzyme. These patterns of nondivergence presumably reflect the "primitive" pattern of gene expression in the ancestral catostomid soon after the polyploidization. Although this pattern seen for some enzymes and tissues in extant species resembles the ancestral pattern, this similarity in expression does not require similar sequences of nucleotides for the regulatory genes. Sequence changes might occur but not result in an alteration in the pattern of gene regulation. It does not appear unreasonable to presume that where patterns of duplicate gene expression are different (within and among tissues) this difference is ultimately due to genetic (nucleotide sequence) divergence.

The Relative Contributions of Mutations at Structural Genes and Regulatory Genes

Unidirectionally divergent gene expression is the most common pattern encountered, with one or more tissues having divergent ratios with the same isozyme predominating in each case. We have found that as an increasing fraction of a set of tissues display divergence in duplicate gene expression, the average divergence ratio within any tissue increases (Fig. 15). This pattern of increase in divergence is also predicted by Zuckerkandl's (1978) controller node stability model.

The patterns of divergent gene expression which fall in the unidirectionally divergent category could be brought about by mutations at structural and/or regulatory genes. A mutation in one of the duplicate structural genes could alter enzyme activity or lability, a change in enzyme activity level which presumably could be tolerated as long as the other isozyme encoded in the other locus is functioning normally. Patterns of divergent gene expression which are similar or identical for all tissues, i.e., have the same ratio of divergence, are likely candidates for structural mutations which reduced the catalytic efficiency of one of the subunit types. Out of all the unidirectionally divergent tissue patterns (84), 12 of these, or 14% could be explained solely by mutational changes at the structural genes. If patterns where the ratio of homopolymer activities of the duplicate genes differed by no more than a factor of two are included, we obtain a more liberal estimate of 23% of the unidirectionally divergent patterns being consistent with a structural gene mutation. However, the above patterns are very likely to include regulatory changes which alter the number of enzyme molecules. The remaining 77% of the divergent patterns of gene expression in the tissues are not easily explained by mutational events occurring solely at structural genes encoding these isozymes.

In most instances of divergent gene expression it is likely that mutations at both structural and regulatory genes are contributing to the differences in duplicate gene expression. A case in point is GPI-A in Fig. 4. A less catalytically active isozyme could have appeared early in catostomid phylogeny, namely the A^2 isozyme, and is now weakly expressed among many taxa compared to the A^1. Subsequent regulatory gene evolution could have modulated the levels of A^1 and A^2 subunits in each tissue, and in one instance for kidney, return to the ratio of 1:1 activity. Alternatively, the kidney could have retained a 1:1 ratio from an ancestral condition with regulatory changes altering the ratios in the other tissues. Obviously, certain patterns can be explained by either a "bidirectional" or "unidirectional" divergence. Regardless of the sequence of

events, most of the divergent gene expressions among the species are sufficiently complex as to suggest a multicomponent process. These patterns of gene expression can also be interpreted in light of Zuckerkandl's (1978) model of gene regulation. As Zuckerkandl (personal communication) has pointed out, most regulatory mutations that occur, and are retained, would be expected to lead to decreases in the affinity between regulator molecules and receptor sequences on the DNA, so that bidirectionality and unidirectionality in regulatory divergence among duplicate isozyme loci could be explained in the following manner. Bidirectionality could occur when a structural gene is controlled by both specific activators and repressors; unidirectionality, when it is controlled by specific activators only. A mutation in an activator system would most often lead to the production of less enzyme, and one in a repressor system, of more enzyme. This concept relates to the control of transcription, but the control of processing of the transcripts could be similarly involved.

Bidirectional patterns of divergence, i. e., predominance of different duplicate genes among different tissues, have probably evolved primarily through regulatory gene changes. Of the total divergent patterns of duplicate gene expression examined, 19% are of this level of complexity. It would be very difficult to explain a complete switch in relative intensities of isozymes from one tissue to another on the basis of differential catabolism. Such an observation is rendered even less likely by the observations of Nadal-Ginard (1978) that the half-lives of the quite structurally divergent LDH isozymes within a tissue are identical.

Perhaps the Ldh-B^1 gene in *Cycleptus elongatus*, shown in Fig. 5, will be lost eventually. The B^1 containing isozymes are weakly expressed in most tissues, and this gene is weakly expressed in heart and gonad compared to the B^2 isozyme. However, a low isozyme activity in a tissue does not automatically mean that the isozyme does not have a crucial metabolic role to play in some cell type.

The Temporal and Spatial Isolation of Duplicate Gene Expression

The temporal and spatial isolation of duplicate gene expression is generally achieved by isozyme systems encoded in duplicate loci considerably older than those we are studying. Because we do not generally observe a departure from the expected binomial isozyme patterns in tissues, we have assumed that in those tissues which are made up of heterogeneous cell populations, the isozyme patterns of each cell type must be very similar (or that the contributions of one cell type are so great that they conceal the contribution of other cell types). We have not examined the contributions of individual cell types to the different isozyme patterns. However, there is some evidence for a spatial isolation of LDH-A gene expression in the intestinal tract of *C. elongatus* (Figs. 8 and 9). Although high levels of both homopolymers are present, the observed amounts of the heteropolymers were far lower than expected. The quite different lactate dehydrogenase A and B gene contributions by different cell types in a tissue have been well documented for kidney of mouse (Nadal-Ginard, 1978). Because the A and B genes have had hundreds of millions of years to evolve such specificities, and because (with the one exception) we have generally observed binomial distributions of isozyme activities, it is likely that the process of regulatory differentiation *among cell types within a tissue or organ* has only just begun in the catostomids, although differentiation of gene expression *among tissues and organs is quite extensive.*

There is little evidence that duplicate genes formed by the recent catostomid tetraploidization have diverged so as to be differentially expressed in development. The only available data are from a study of the isozymes of the catostomid *Erimyzon sucetta* (Shaklee et al., 1974). There was no indication of differential temporal expression of the duplicate loci but the sample of enzymes was small and further developmental analyses are required to determine the extent of divergence at this level in 50 million years.

Relationship of Extent of Divergence of Duplicate Gene Expression and the Level of Morphological Advancedness

The present investigation of differential gene expression of species belonging to many genera provides an evolutionary dimension to the study of duplicate gene expression. The lowest average divergence for all tissues within a species is exhibited by the morphologically primitive *Ictiobus bubalus*, 1:1.18, and the greatest divergence is shown by the advanced *Catostomus discobolus*, 1:2.37. Two species within the primitive subfamily Ictiobinae, *I. bubalus* and *C. cyprinus*, possess quite different extents of divergence of duplicate genes, the former close to the ancestral condition with an average ratio of 1:1.2, and the other quite evolved, with an average ratio of 1:2.0. Thus, some primitive species have retained ancestral patterns of gene regulation; some have not, a phenomenon noted also by Fisher and Whitt (1978) for genes duplicated early in vertebrate evolution. No correlation of extent of divergence of duplicate genes in a species was detected with the fraction of duplicate genes functional (a measure of "primitiveness").

Differences in the tissue patterns of duplicate gene expression, which exist *among* different species, might first arise as a polymorphism of gene regulation *within* a species. We did find a slight variation in the ratios of duplicate gene expression among individuals of *C. cyprinus*. However, it would be premature to ascribe this variation among individuals of a species as due to polymorphisms at regulatory genes until we are able to exclude the possibility that these differences arose either artifactually or through alterations at the epigenetic level. In any event, the low variances in the ratios of duplicate gene expression for the four enzymes studied suggest that regulatory polymorphisms, if present, are not causing substantial changes in the tissue patterns of differential gene expression as visualized by staining intensities on the gel.

A trend exists for increasing differences in the patterns of divergence with increasing taxonomic distance, when one compares only homologous enzyme systems between species pairs (Table 5). Closely related species have similar patterns, and thus a low index of divergence. More distant species share fewer patterns in common and have higher indices of divergence. The two species mentioned above in the Ictiobinae which exhibited quite different indices of divergence are separated by a considerable genetic distance (Ferris et al., 1979) and have many differences in duplicate genes expressed.

If disomy is achieved rapidly and at comparable times for all chromosomes, the divergence of duplicate gene expression may serve as the basis for a crude "regulatory clock". Such patterns of change would be consistent with either a series of neutral mutational events or with mutational events having a slight functional impact, yet largely selected. These results may also be used to support the "genetic sufficiency" proposal of Zuckerkandl (1978).

Relationships Between the Divergence of Gene Expression and the Structure and Function of the Enzymes

The 15 enzymes studied exhibited a wide range of divergences for their duplicate genes. Is the divergence of duplicate gene expression within tissues related to the structural and functional properties of the isozymes? First, the average ratio of duplicate gene expression was determined for each enzyme over all the species. The average ratios were then compared with the average heterozygosity of the enzymes in the duplicate state among catostomids (Table 6). No correlation was found. These results suggest that the mutational process affecting net charge is independent of the mutational process affecting the divergence of duplicate gene expression, the latter generally due to mutations at regulatory genes. An uncoupling of structural and regulatory gene evolution has been postulated by Zuckerkandl and Pauling (1965), Zuckerkandl (1968), King and Wilson (1975), and Cherry et al. (1978). This postulate is further supported by an absence of correlation of the divergence of duplicate genes with the subunit molecular weight of the isozymes they encoded.

Another property of the isozymes, net charge, is nonrandomly associated with the relative predominance of the duplicate gene expressions. Specifically, when there is a divergent ratio within a tissue, the more anodally migrating isozyme is more likely to predominate over the less anodally migrating isozymes. Dice and Goldberg (1975) reported that relatively acidic proteins are more susceptible to degradation than basic proteins. Since, in our study, it is the more basic proteins which are relatively diminished, it is unlikely that degradation is a prime factor in generating the divergent ratios we have found. In any event, the relative electrophoretic mobilities of such divergent isozymes as the LDH-A_4 and B_4 are often reversed in fish, which suggests that there is not a close association of net charge and tissue predominance of isozymes (Markert et al. 1975). One possible explanation for the tendency of the more anodal isozymes to predominate in their tissue expression is that early in phylogeny, perhaps in one or few species, this predominance arose (by chance?) and has been subsequently distributed among the many species derived from the ancestral ones. Further studies will be needed to determine what, if any, relationship exists between enzyme stability, function, and net charge.

One measure of the functional specificity of an enzyme is the number of tissues in which it predominates. We found no correlation of the degree of divergence of expression of duplicate loci and the number of tissues in which the enzyme activity predominates. These various lines of evidence are consistent with the model that the initial appearance of tissue specificity of these isozymes is established by random mutations at regulatory genes, proposed by Zuckerkandl (1978).

Relationships of Tissue Physiology and Cell Division to the Extent of Divergence of Duplicate Gene Expression

The study of different adult tissues provides a developmental and physiological dimension to the study of differential gene expression. If the amount of divergence in each tissue is averaged over the 15 species and 15 enzymes, there is a definite tissue effect upon the extent of divergence of expression of duplicate genes. As illustrated for mitochondrial MDH in Fig. 3 slight divergence has occurred in brain and muscle of the

H. nigricans and rather extreme divergent ratios are present in stomach and liver. Because the differences in the rates of protein synthesis (and degradation) among these tissues correspond approximately with the extent of divergence of duplicate gene expression within threse tissues, a causal relationship may exist. It should be emphasized that in these adult tissues there are almost certainly "steady state" conditions obtaining which reflect a balance between isozyme synthesis and degradation. Therefore tissues with low rates of enzyme catabolism have low rates of synthesis (e.g., brain). In tissues where there is rapid degradation of enzymes (e.g., liver) this is balanced by a rapid synthesis of these enzymes to maintain a steady state. Because of this confounding it is difficult to determine whether either process by itself is an important determinant or selective force for the establishment of the divergent patterns. Since levels of protein synthesis and turnover are also a partial measure of the general cellular metabolism, perhaps some component of cellular metabolism is associated with the extent of divergence of duplicate gene expression.

A measure of the degree of differentiation of a tissue is the mitotic activity of a cell, since it is observed that highly differentiated cells undergo essentially no mitoses (Bullough, 1965). In the catostomids the tissues with no or low mitosis (brain, eye, muscle) have the least divergent ratios of duplicate gene expression, whereas the tissues exhibiting considerable mitotic activity (stomach and liver) have the most divergent ratios.

The extent of divergence of duplicate gene expression in a tissue may also be related to the degree of homeostasis of that tissue. The homeostatic requirements of brain and muscle are probably greater than those for stomach or liver. Indeed, brain and muscle appear in development prior to stomach and liver (Long and Ballard, 1976). Further studies on the evolutionary changes in enzyme amounts and activity as a function of gene dosage are clearly needed.

The Relative Contributions of Isozyme Synthesis and Catabolism to the Patterns of Divergent Gene Expression

To what extent are the different ratios of isozymes within and among tissues brought about by differential gene regulation, i.e., different rates of transcription or different levels of transcripts? There is recent evidence from studies of lactate dehydrogenase and other proteins from higher vertebrates that differences in the isozyme ratios *within tissues* are the consequence of differential synthesis and not differential catabolism (Nadal-Ginard, 1978). It is established that the steady state levels of total enzyme activity in developmental stages (Gilberg and Johnson, 1972) or within adult differentiated tissues (Majerus and Kilburn, 1969) is a balance between the rate of protein synthesis and degradation. Differential degradation of related isozymes can occur within a cell, as shown for different isozymes of catalase (Jones and Masters, 1974). However, these two forms of catalase have quite different subcellular locations, the cytosol and peroxisomes. Isotope labelling of proteins in tissues of mice has revealed that different *levels* of total lactate dehydrogenase *among tissues* are determined primarily by differences in enzyme catabolism. More importantly, Nadal-Ginard found that *within* any given tissue of the mouse, LDH-A and LDH-B subunit containing isozymes are degraded at the same rate. Thus, it was concluded that different ratios of A and B isozymes in the various tissues must be due to different rates of protein synthesis, presumably

reflecting differences in transcription. The same conclusion was reached for the aldolase isozymes in tissues of rabbit (Lebherz, 1975).

There is some direct experimental evidence for differential gene regulation in another tetraploid group, the salmonid fishes. Duplicate LDH-A loci in salmon produce subunits which are in the ratio of 4:1 (Lim and Bailey, 1977), yet examination of their kinetic properties revealed no differences in catalytic efficiency of the two forms. Thus it was hypothesized that the nonequivalent expression of these duplicate genes is due to different rates of synnthesis.

On the basis of the kinds of patterns of duplicate gene expression we have found, as well as of studies on enzyme degradation and catalytic efficiency in other organisms, we propose that most if not all the tissue characteristic isozyme patterns are brought about at the level of enzyme synthesis and most probably the level of transcription or processing of the nuclear RNA transcript. As indicated before, some duplicate genes are so divergent in their expression that only one isozyme is detected in a tissue, e.g., CK-B_2^2 in heart in Fig. 6. This variable presence or absence of a specific gene function in a tissue over many taxa is analogous to the situation described for the taxonomically variable Ldh-B gene activity in erythrocytes of rodents (Shows et al. 1969). Since most rodents have LDH-B activity in erythrocytes, and myomorph rodents lack the LDH-B activity in erythrocytes but have B gene activity in other tissues, a tissue-specific defect in gene regulation was postulated to have evolved in the evolution of this taxon. These examples provide further support for a regulatory basis for many of the patterns of differential expression in catostomids.

Phylogenetic Analysis of the Probable Times of Initiation of Divergence in Duplicate Gene Expression

The probable phylogenetic times at which several of the tissue patterns of duplicate gene expression arose were able to be determined. The patterns of CK-B expression (see Figs. 6 and 17) appear to have evolved soon after the polyploidization event, perhaps within 5-10 million years after gene duplication. It is possible, but much less likely, that the many unrelated species have arrived independently at the pattern by convergence. A similar early evolutionary appearance of a bidirectionally divergent pattern was demonstrated in the tetraploid cobitid fishes for duplicate LDH-B loci (Ferris and Whitt, 1977b). Other proteins, for example, MDH and GPI, were shown to have evolved duplicate gene divergence somewhat later in the phylogeny of the catostomids. The regulatory elements for these enzymes might be evolving more slowly than other enzymes we studied. For example, the structural genes of other enzymes, such as LDH-B, appear to be associated with regulatory genes readily capable of evolving new tissue specificities since tissue divergence of LDH-B locus expression has been found in salmonids (Holmes and Markert, 1969; Lim et al., 1975), catostomids, cobitids (Ferris and Whitt, 1977b) and rodents (Shows et al., 1969).

The widespread pattern of differential CK-B locus expression in catostomids, and the retention of duplicate CK-B loci in the 30 diverse catostomids examined, raises an interesting question. Are these duplicates being maintained because they have diverged functionally in their kinetic properties, so that each form is advantageous in a unique tissue? Zuckerkandl (1978) has recently proposed that differential expression among tissues following gene duplication is brought about by mutations in

associated regulatory elements. Much of our data from the catostomids is consistent with this theory, and it may well describe divergence in early phases of gene duplication. Petit and Zuckerkandl (1976) suggest that a duplicate gene function might be eliminated soon after duplication if it fails to acquire any unique functional or tissue specificity. The fact that enzymes such as CK-B and SOD have been retained, in a duplicate gene set in all catostomids, often with different electrophoretic mobilities and have divergent patterns of expression in most species, suggests that they may have acquired functional (but not necessarily kinetic) differences and thus are currently resisting genic diploidization. An investigation of the microlocalizations and the kinetic and physical properties of the isozymes encoded in these duplicate loci are necessary to determine whether a functional divergence of these isozymes has accompanied the regulatory divergence of their loci. We further suggest that once a duplicate gene pair has evolved, tissue-specific patterns of predominance, especially the bidirectional tissue patterns, and kinetic differences (each consistent with the appropriate cellular environment) may be selected and thus the duplicate loci will tend to be retained in the genome. Indeed, ancient duplicate copies of LDH, MDH, and CK genes in the teleosts have been maintained is almost all teleost lineages, and usually each duplciate is differentially expressed in characteristic tissues over most of the taxa (Markert et al., 1975; Fisher and Whitt, 1978; Fisher et al., 1979).

Future Fates of Duplicate Gene Expression in the Catostomids

A study of the kinetic properties of the isozymes encoded in the LDH-A and B loci in a number of vertebrate species, including fishes, reveals that the A_4 isozymes have affinities for pyruvate which are 2-10 times greater than the B_4 isozymes (Pesce et al., 1967). The relative difference in this kinetic property presumably arose in the common ancestor of all the species, which was about 425 million years ago (Dayhoff, 1972). The duplication of LDH to give rise to A and B genes is thought to have occurred 450 million years ago in the ancestors of the Myxiniformes (Markert et al., 1975). The kinetic properties of duplicate LDH-B isozymes in trout have also been found to be significantly different, and this difference was postulated to have arisen in about 100 million years (Lim et al., 1975), and is paralleled by a regulatory divergence in the expression of the two loci (Holmes and Markert, 1969). Therefore, functional specialization of the isozymes may have been acquired 24-100 million years after the acquisition of tissue specificity of duplicate gene expression, or the regulatory and functional specializations may have coevolved.

We do not know what fraction of the remaining duplicate genes will be lost in the next 50 million years. However, based on the relatively large number of multilocus isozyme systems remaining in diploid vertebrates (many of the isozyme loci formed by polyploidization 500 million years ago), it is likely that the rate of loss of duplicate gene expression will be slower in the future than it has been in the past. Some tissue patterns of duplicate gene expression may allow a duplicate gene expression to be lost more readily than others. If two duplicate genes are simultaneously expressed in the same tissues, and are functionally redundant, one of these locus expressions will be evolutionarily dispensable. The probability of this loss being established in a species would be expected to depend upon the extent to which the duplicate genes have functionally diverged and the population size. The Ldh B^1 gene of *C. elongatus* in Fig. 5

is a possible candidate for this loss. It is weakly expressed in most tissues and is strongly expressed in some tissues in which another, functionally and structurally very similar, duplicate gene is also strongly expressed. If the diminished product of the Ldh-B^1 locus is not essential, it could be "silenced" in the future through the formation and fixation of a null allele. In this context, it is interesting to note that LDH-B has undergone genic diploidization in some taxa (Ferris and Whitt, 1978a) and is perhaps presently doing so in tetraploid carp (Engle et al., 1973). A hypothesis similar to ours has been advanced for the duplicate LDH-A loci is salmonids (Lim and Bailey, 1977).

If highly divergent duplicate locus expression is selected for, loss of a duplicate gene should be resisted. One would expect to detect a relationship between the fraction of species in which an enzyme has been silenced and the average extent of divergence of duplicate gene expression in species which have retained both copies of the gene. The absence of a significant inverse correlation between *loss* of duplicate gene expression and *extent of divergence of expression*, Table 6, suggests that many of the divergent patterns of duplicate gene expression may not, at present, be functionally divergent and thus not strongly selected for (Petit and Zuckerkandl, 1976). This suggestion does not preclude the fact that some of the duplicate genes have evolved new tissue specific functions. Duplicate genes which have diverged in their expression in a large number of taxa, e.g., CK-B, SOD, and M-MDH may have reached this stage. Tissue-specific isozymes with significant kinetic differences, encoded in duplicate loci formed much earlier than the relatively recently duplicated loci, have been found to persist in many taxa of vertebrates. These include muscle, liver, and retinal-specific LDHs (Whitt et al., 1973; Shaklee et al., 1973; Markert et al., 1975), testes-specific creatine kinases (Eppenberger et al., 1971; Fisher and Whitt, 1978), and LDHs (Goldberg, 1977) and cytochrome c's (Goldberg, 1977) as well as many others. However, the fact that the catostomids are relatively recent polyploids, with approximately 50% of their duplicate genes still expressed, suggests that further loss of duplicate gene expression will probably occur.

Future research is required to focus on the relative contributions of regulatory gene evolution, and isozyme structural and functional evolution in the early stages of gene duplication (zero to 100 million years after the transition to disomy). Many of the divergent patterns of gene expression in catostomids will tend to persist with the duplicate genes becoming increasingly restricted in their spatial and temporal expression, as have many of the multilocus systems in vertebrates formed by more ancient gene duplications. The continued investigation of tissue expression of duplicate genes relatively soon after gene duplication should provide insights into the mechanisms responsible for differential gene regulation and the evolutionary forces responsible for the establishment of these temporal and spatial specificities of gene function.

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Appendix

The ratios of duplicate gene expression in tissues of 15 catostomids are shown in Table 10 below. The tissues examined are white skeletal muscle (M), heart (H), eye (E), brain

(B), stomach (S), gill (G), liver (L), spleen (Sp), gonad (Go), and kidney (K). The first row of values for an enzyme is the estimated ratio of subunits encoded by the duplicate genes. The second row is the logarithm of these ratios. A minus (-) sign before the log value indicates that the less anodally migrating isozyme predominated in its expression over the more anodally migrating isozyme. No sign indicates that the more anodally migrating isozyme predominated. A blank indicates no enzyme activity was detected. In the calculation of the means for enzymes and tissues, the minus (-) signs were omitted.

The ratio of activities of the duplicate genes determined by the method of Klebe (1975) are denoted by an asterisk (*). A replication of the determination of the ratio of duplicate gene expression was performed in some instances and these are indicated by a plus (+) sign.

Table 10. *Cycleptus elongatus*

	Tissues									
Enzyme	M	H	E	B	S	G	L	Sp	Go	K	Mean (log)
Acp		1	1	1	1	1	1.41*	1	1	1	
		0	0	0	0	0	-0.15	0	0	0	0.02
Gpi-A		5.66	16	5.66	16	16	8	16	8	16	
		0.75	1.20	0.75	1.20	1.20	0.90	1.20	0.90	1.20	1.04
Gpi-B	2.83*										
	-0.45										0.45
M-mdh	1.41+	1	1	1	2	2	4	4	4	4*	
	0.15	0	0	0	0.30	0.30	0.60	0.60	0.60	0.60	0.32
Mdh-A		1	1	1	1	1	1	1	2	1*	
		0	0	0	0	0	0	0	-0.30	0	0.03
Mdh-B	1*+	1									
	0	0									0.00
Ldh-A	1*+	2.83	2.83	2.83	1.68*+	2.83*	4	4	4	3.36*	
	0	0.45	0.45	0.45	-0.23	0.45	0.60	0.60	0.60	0.53	0.44
Ldh-B		2.83	3.36	2.83	4	1.68	1.68	4	2	2.83	0.44
		-0.45	-0.53	-0.45	-0.60	-0.23	-0.23	-0.60	-0.30	-0.45	
Ck-A	1*+	1									
	0	0									0.00
Ck-B		16	5.66*	1*							
		-1.20	0.75	0							0.65
G3pdh	1*										
	0										0.00
Ak-A	1*	1	1	1		1	16*	1	1	1	
	0	0	0	0		0	-1.20	0	0	0	0.14
Sod		1	1	1.41	1	1	2*	2*	1*	1	
		0	0	-0.15	0	0	0.30	-0.30	0	0	0.08
Mean (log)	0.09	0.26	0.33	0.20	0.33	0.27	0.50	0.41	0.34	0.35	

Mean of all tissues, enzymes = 0.31 (log), 1:2.03 (inverse log)

Tab. 10. (cont.) *Ictiobus bubalus*

Enzyme	Tissues										Mean (log)
	M	H	E	B	S	G	L	Sp	Go	K	
Acp		1	1	1	1	1	1*	1	1	1	
		0	0	0	0	0	0	0	0	0	0.00
Gpi-A		1	1	1	1	1	1	1	1	1	
		0	0	0	0	0	0	0	0	0	0.00
Gpi-B	1*	1									
	0	0									0.00
M-mdh	2.83*	1	1	1*	1.41	1	1.41	2	2.83	1	
	0.45	0	0	0	0.15	0	0.15	0.30	0.45	0	0.14
Ck-A	1*	1									
	0	0									0.00
Ck-B		16	4*	1*							
		-1.20	0.60	0							0.60
G3pdh	1*										
	0										0.00
Ak-A	1*	1	1	1	1	1	1	1	1	1	
	0	0	0	0	0	0	0	0	0	0	0.00
Sod		1	1	1	1	1	1	1	1	1	
		0	0	0	0	0	0	0	0	0	0.00
6Pgd		1	1	1	1.41	1.41*	1*	1	1	1.41	
		0	0	0	-0.15	-0.15	0	0	0	-0.15	0.05
Ald-C			2.83	2.83							
			-0.45	-0.45							0.45
Mean (log)	0.09	0.13	0.13	0.06	0.05	0.03	0.03	0.05	0.08	0.03	

Mean of all tissues, enzymes = 0.07 (log), 1:1.18 (inverse log)

Tab. 10. (cont.) *Carpiodes cyprinus*

Enzyme	Tissues M	H	E	B	S	G	L	Sp	Go	K	Mean (log)
Sod	16	16	2	2	2.83	2*	2+	2	1*	4*	
	1.20	1.20	0.30	0.30	0.45	0.30	0.30	0.30	0	0.60	0.50
Ldh-B		1.68*	4	4	4	4	4	4	1.68	4	
		0.23	0.60	0.60	0.60	0.60	0.60	0.60	0.23	0.60	0.52
Mdh-B	1+										
	0										0.00
Ak-A	1	1*	1	1	1	2	1	1	1	1	
	0	0	0	0	0	-0.30	0	0	0	0	0.03
G3pdh	1*										
	0										0.00
Ald-C			2.83	2.83							
			-0.45	-0.45							0.45
6Pgd		1	1	1.41	1.41	2*	1*	1	2	1.41	
		0	0	-0.15	-0.15	-0.30	0	0	-0.30	-0.15	0.12
Gpi-A		2	2*	2*+	2	2	2.83*+	2	2	5.66*+	
		-0.30	-0.30	-0.30	-0.30	-0.30	-0.45	-0.30	-0.30	-0.75	0.37
Gpi-B	1*	2		16	1				1	1	
	0	-0.30		1.20	0				0	0	0.25
Ck-B		16	2.83*+	1*+							
		-1.20	0.45	0							0.55
Mean (log)	0.24	0.46	0.30	0.38	0.25	0.36	0.27	0.24	0.14	0.35	

Mean of all tissues, enzymes = 0.31 (log), 1:2.02 (inverse log)

Tab. 10. (cont.) *Erimyzon sucetta*

Enzyme	Tissues M	H	E	B	S	G	L	Sp	Go	K	Mean (log)
Ck-B		16	1.41*+								
		-1.20	0.15	0							0.45
M-mdh	1.41*	1	1	1	1.41+	2.83	2.83	2.83	2	2.83	
	-0.15	0	0	0	-0.15	-0.45	-0.45	-0.45	-0.30	-0.45	0.24
Mdh-B	4*										
	-0.60										0.60
Ak-A	1*	1	1	1	1	1	1	4*	1	4	
	0	0	0	0	0	0	0	-0.60	0	-0.60	0.12
Sod	2	2	1.41	2	2	2	2	2	2*	2	
	-0.30	-0.30	-0.15	-0.30	-0.30	-0.30	-0.30	-0.30	-0.30	-0.30	0.29
Ldh-B		2	2.83	2.83		2.83		4	1	3.36	
		-0.30	-0.45	-0.45		-0.45		-0.60	0	-0.53	0.40
Mean (log)	0.26	0.36	0.15	0.15	0.15	0.30	0.25	0.50	0.15	0.47	

Mean of all tissues, enzymes = 0.27 (log), 1:1.88 (inverse log)

Tab. 10. (cont.) *Erimyzon oblongus*

Enzyme	Tissues M	H	E	B	S	G	L	Sp	Go	K	Mean (log)
Ck-B			1.41	1.41							
			0.15	-0.15							0.15
Mdh-B	2.83										
	-0.45										0.45
M-mdh	1.41	1	1	1	5.66	1	2	2	2	1.41	
	-0.15	0	0	0	0.75	0	0.30	0.30	0.30	0.15	0.20
Ak-A	1	1	2	1	2	1	2	8	8	8	
	0	0	-0.30	0	-0.30	0	-0.30	-0.90	-0.90	-0.90	0.36
Sod	1	1	1	1	1	1	2	1.41	1	1	
	0	0	0	0	0	0	-0.30	0.15	0	0	0.05
Ldh-B		2	2.83	2.83	4	2.83		4	4	3.36	
		-0.30	-0.45	-0.45	-0.60	-0.45		-0.60	-0.60	-0.53	0.50
6Pgd		1	1	1	1.41	2	1	1	1.41	1	
		0	0	0	0.15	0.30	0	0	0.15	0	0.07
Gpi-B	2	2			2						
	0.30	0.30			0.30						0.30
Mean (log)	0.18	0.10	0.15	0.10	0.35	0.15	0.23	0.39	0.39	0.32	

Mean of all tissues, enzymes = 0.23 (log), 1:1.70 (inverse log)

Tab. 10. (cont.) *Erimyzon tenuis*

Enzyme	Tissues M	H	E	B	S	G	L	Sp	Go	K	Mean (log)
Ck-B			1.41	1							
			0.15	0							0.08
Mdh-B	4										
	-0.60										0.60
M-mdh	1	1.41	1	1	2	1	4	4	4	1.41	
	0	0.15	0	0	0.30	0	0.60	0.60	0.60	0.15	0.24
Sod		1	1	1	1.41	1	2	1.41	4	1	
		0	0	0	-0.15	0	-0.30	0.15	-0.60	0	0.13
Ldh-B		1	1.41	1.41	1	1.19		4	1	2.83	
		0	-0.15	-0.15	0	-0.08		-0.60	0	-0.45	0.18
Gpi-B	1.41	1.41	1						1.41	1.41	
	0.15	0.15	0						0.15	0.15	0.12
Mean (log)	0.25	0.08	0.06	0.04	0.15	0.03	0.45	0.45	0.34	0.19	

Mean of all tissues, enzymes = 0.18 (log), 1:1.52 (inverse log)

Tab. 10. (cont.) *Minytrema melanops*

Enzyme	Tissues M	H	E	B	S	G	L	Sp	Go	K	Mean (log)
Gpi-A		2	2	1	1.41	1.41	4*	1	4	1.41*+	
		0.30	0.30	0	0.15	0.15	0.60	0	0.60	0.15	0.25
Gpi-B	1	1.41		2	2				1	1	
	0	-0.15		-0.30	-0.30				0	0	0.13
M-mdh	1	1	2	1	1.41	1	2	1.41	2	1	
	0	0	0.30	0	0.15	0	0.30	0.15	0.30	0	0.12
Mdh-B	4										
	-0.60										0.60
Ck-B			1.41	1							
			0.15	0							0.08
Ald-C			1.68	1							
			-0.23	0							0.11
Sod		1	1.41	1	1.41	2.83	1.41*	16	2	4*	
		0	0.15	0	0.15	0.45	0.15	1.20	-0.30	0.60	0.33
Mean (log)	0.20	0.11	0.23	0.05	0.19	0.20	0.35	0.45	0.30	0.19	

Mean of all tissues, enzymes = 0.22 (log), 1:1.64 (inverse log)

Tab. 10. (cont.) *Moxostoma duquesnei*

Enzyme	Tissues M	H	E	B	S	G	L	Sp	Go	K	Mean (log)
Sod	1.41	1	1	1	1.41	2.83*	1*	1	4	2	
	0.15	0	0	0	0.15	0.45	0	0	-0.60	0.30	0.17
Ck-B			2*	1*							
			0.30	0							0.15
Gpi-A	2	5.66	4*	1.41*	4	2.83	2	2	2	1.41	
	0.30	0.75	0.60	0.15	0.60	0.45	0.30	0.30	0.30	0.15	0.39
Ak-A	1*	1	1	2*	16	1	32	1	1	1	
	0	0	0	-0.30	-1.20	0	-1.51	0	0	0	0.30
Mdh-A		1	1	1*+	4*	2.83*	2	4	4	4	
		0	0	0	-0.60	-0.45	-0.30	-0.60	-0.60	-0.60	0.35
M-mdh	1.41	2	1	1*	4	2	11.3*	1	8	1.41	
	0.15	0.30	0	0	0.60	0.30	1.05	0	0.90	0.15	0.35
Ldh-B		1	2.83	1.19	1	3.36	4	4	1.41	1.19	
		0	0.45	0.08	0	0.53	0.60	0.60	0.15	0.08	0.28
Mean (log)	0.15	0.18	0.19	0.08	0.53	0.36	0.63	0.25	0.43	0.29	

Mean of all tissues, enzymes = 0.30 (log), 1:1.99 (inverse log)

Tab. 10. (cont.) *Moxostoma erythrurum*

Enzyme	Tissues M	H	E	B	S	G	L	Sp	Go	K	Mean (log)
Sod	2	1	1	1	1.41	2.83	1	1	2	1	
	0.30	0	0	0	0.15	0.45	0	0	-0.30	0	0.12
Ck-B			2	1							
			0.30	0							0.15
Gpi-A	2	2.83	2.83	1	1.41	1.41	2	2	2.83	1.41	
	0.30	0.45	0.45	0	0.15	0.15	0.30	0.30	0.45	0.15	0.27
Ak-A	1	1	1	1		2	1	1	1	1	
	0	0	0	0		0.30	0	0	0	0	0.03
Mdh-A		1	1	1	2.83	2.83	2.83	2.83	1	2.83	
		0	0	0	-0.45	-0.45	-0.45	-0.45	0	-0.45	0.25
M-mdh	2	1	1	1	5.66	1.41	5.66	4	1.41	1	
	0.30	0	0	0	0.75	0.15	0.75	0.60	0.15	0	0.27
Mean (log)	0.23	0.09	0.13	0.00	0.38	0.30	0.30	0.27	0.18	0.12	

Mean of all tissues, enzymes = 0.19 (log), 1:1.55 (inverse log)

Tab. 10. (cont.) *Hypentelium nigricans*

Enzyme	Tissues M	H	E	B	S	G	L	Sp	Go	K	Mean (log)
G3pdh	8										
	-0.90										0.90
Gpi-A		5.66	5.66*	2.83*	2.83	4	2.83	4	2	1	
		0.75	0.75	0.45	0.45	0.60	0.45	0.60	0.30	0	0.48
6Pgd		1.41	1	1.41	2	1	1	1	1	1	
		0.15	0	-0.15	0.30	0	0	0	0	0	0.07
Sod	1.41	1.41	1	1.41	1.41	1.41	2*+	1	1.41	2	
	0.15	0.15	0	-0.15	0.15	0.15	0.30	0	-0.15	0.30	0.15
Mdh-A		1	1	1*	2	1.41	4*	2.83	1	2.83	
		0	0	0	-0.30	-0.15	-0.60	-0.45	0	-0.45	0.22
M-mdh	2.83	1.41	1.41	1*	1.41	1	8*	1.41	5.66	1.41	
	0.45	0.15	0.15	0	0.15	0	0.90	0.15	0.75	0.15	0.29
Ck-B			2.83	1							
			0.45	0							0.23
Mean (log)	0.50	0.24	0.23	0.13	0.27	0.18	0.45	0.24	0.24	0.18	

Mean of all tissues, enzymes = 0.25 (log), 1:1.79 (inverse log)

Tab. 10. (cont.) *Catostomus commersoni*

Enzyme	Tissues										Mean (log)
	M	H	E	B	S	G	L	Sp	Go	K	
Ck-B		16	2*	1*							
		-1.20	0.30	0							0.50
Ldh-B		2.83	2.83	2.83	4	4	4	4	1	3.36	
		0.45	0.45	0.45	0.60	0.60	0.60	0.60	0	0.53	0.48
Gpi-A		5.66	11.3*	5.66*	11.3	4	4	8	1	2.83*	
		0.75	1.05	0.75	1.05	0.60	0.60	0.90	0	0.45	0.69
6Pgd	1	1	1.41	2	1.41	1	1*	1	1.41	1	
	0	0	0.15	0.30	-0.15	0	0	0	-0.15	0	0.08
G3pdh	4*										
	-0.60										0.60
Mdh-A	2	2.83	1.41	1*	1.41	2.83	2.83	2	4	1	
	0.30	0.45	0.15	0	0.15	0.45	0.45	0.30	0.60	0	0.29
M-mdh	1	1	1	1*	2.83	1	1.41*	1.41	2	2	
	0	0	0	0	0.45	0	0.15	0.15	0.30	0.30	0.14
Sod		1	1	1	2	2	2*	2	1	2*	
		0	0	0	0.30	0.30	0.30	0.30	0	0.30	0.17
Mean (log)	0.23	0.41	0.30	0.21	0.45	0.33	0.35	0.38	0.18	0.26	

Mean of all tissues, enzymes = 0.32 (log), 1:2.08 (inverse log)

Tab. 10. (cont.) *Catostomus catostomus*

Enzyme	M	H	E	B	S	G	L	Sp	Go	K	Mean (log)
Ak-A	1	1	1	1	1	1	1	1	1	1	
	0	0	0	0	0	0	0	0	0	0	0.00
Ldh-B		2.38	4	2.83	4	2.83		4	1	3.36	
		0.38	0.60	0.45	0.60	0.45		0.60	0	0.53	0.45
M-mdh	4	4	5.66	2.83		5.66	5.66	5.66	5.66	5.66	
	-0.60	-0.60	-0.75	-0.45		-0.75	-0.75	-0.75	-0.75	-0.75	0.69
Mdh-A	1	2.83	1	1	1	1.41	1.41	2	3	1.41	
	0	0.45	0	0	0	0.15	0.15	0.30	0.30	0.15	0.15
Sod	2	2	2.83	1.41	1.41	2	2.83	2.83	1	4	
	-0.30	-0.30	-0.45	-0.15	-0.15	0.30	-0.45	-0.45	0	-0.60	0.32
Gpi-A	5.66	5.66	8	5.66	5.66	5.66	5.66	4	4	2	
	0.75	0.75	0.90	0.75	0.75	0.75	0.75	0.60	0.60	0.30	0.69
Gpi-B	1	1									
	0	0									0.00
Ck-B		16	1.41	1							
		-1.20	0.15	0							0.45
6Pgd	1.41	1	2.83	1	1.41	1	1	1	1.41	1	
	-0.15	0	0.45	0	-0.15	0	0	0	-0.15	0	0.09
Mean (log)	0.26	0.41	0.41	0.26	0.28	0.34	0.35	0.39	0.26	0.33	

Mean of all tissues, enzymes = 0.33 (log), 1:2.12 (inverse log)

Tab. 10. (cont.) *Catostomus discobolus*

Enzyme	Tissues M	H	E	B	S	G	L	Sp	Go	K	Mean (log)
Ak-A	1	1	1	1		1	1	1	1	1	
	0	0	0	0		0	0	0	0	0	0.00
Mdh-A		2.83	2.83	2.83	2.83	2	2.83	2	2	1.41	
		0.45	0.45	0.45	0.45	0.30	0.45	0.30	0.30	0.15	0.37
M-mdh	16	1	1	2		2.83	16	1.41	2.83	16	
	-1.20	0	0	-0.30		-0.45	-1.20	-0.15	-0.45	-1.20	0.55
6Pgd	16	5.66	5.66	5.66	1.41	1.41	5.66	1	5.66	16	
	-1.20	-0.75	-0.75	-0.75	-0.15	-0.15	-0.75	0	-0.75	-1.20	0.65
Sod	1	1	1	2	1.41	1	1	1	1.41	1.41	
	0	0	0	-0.30	-0.15	0	0	0	-0.15	-0.15	0.08
Ldh-B		1.19	2.83	1.41	2.83	1.19		3.36	1.19	3.36	
		0.08	0.45	0.15	0.45	0.08		0.53	0.08	0.53	0.29
Gpi-A		5.66	5.66	5.66	4	5.66	5.66	4	4	1.41	
		0.75	0.75	0.75	0.60	0.75	0.75	0.60	0.60	0.15	0.64
Ck-B		16	2	1							
		-1.20	0.30	0							0.50
Mean (log)	0.60	0.40	0.34	0.34	0.36	0.25	0.53	0.23	0.33	0.48	

Mean of all tissues, enzymes = 0.37 (log), 1:2.37 (inverse log)

Tab. 10. (cont.) *Chasmistes brevirostris*

Enzyme	Tissues M	H	E	B	S	G	L	Sp	Go	K	Mean (log)
Ak-A	1 0	1 0	1 0	1 0		2 0.30	1 0	1 0	1 0	1 0	0.03
Ck-B		16 -1.20	1.41 0.15	1 0							0.45
Gpi-A	2.83 0.45	5.66 0.75	4 0.60	5.66 0.75		5.66 0.75		4 0.60	5.66 0.75	4 0.60	0.66
Gpi-B	2.83 -0.45	2.83 -0.45									0.45
Ldh-B		2.38 0.38	4 0.60	2.38 0.38	4 0.60	3.36 0.53		4 0.60		3.36 0.53	0.52
Mdh-A		2 0.30	1.41 0.15	1 0	1 0	1.41 0.15	1.41 0.15	1.41 0.15	1.41 0.15	1 0	0.12
M-mdh	4 -0.60	4 -0.60	1 0	2.83 -0.45		4 -0.60	4 -0.60	16 -1.20	5.66 -0.75	16 -1.20	0.67
6Pgd		2 0.30	2 0.30	1 0	1 0	1.41 0.15	2 0.30	1.41 0.15	1 0	2 0.30	0.17
Sod	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	1 0	0.00
Mean (log)	0.30	0.44	0.23	0.20	0.15	0.35	0.21	0.39	0.28	0.38	

Mean of all tissues, enzymes = 0.30 (log), 1:2.01 (inverse log)

Tab. 10. (cont.) *Xyrauchen texanus*

Enzyme	Tissues M	H	E	B	S	G	L	Sp	Go	K	Mean (log)
Ak-A	1	1	1	1	1	1	1	-	1	1	
	0	0	0	0	0	0	0		0	0	0.00
Ck-B			1.41	1							
			0.15	0							0.08
Gpi-A		8	8	8	16	8	8	-	8	8	
		0.90	0.90	0.90	1.20	0.90	0.90		0.90	0.90	0.94
Gpi-B	2	1						-			
	-0.30	0									0.15
G3pdh	5.66							-			
	-0.75										0.75
Ldh-B		2.83	2.83	2.83	4	1.68		-	4	1.68	
		0.45	0.45	0.45	0.60	0.23			0.60	0.23	0.43
Sod	1	1	1	1.41	1	1	2	-		1	
	0	0	0	-0.15	0	0	0.30			0	0.06
M-mdh	1	1.41	1	1	1.41	1	2	-	1	1	
	0	0.15	0	0	-0.15	0	-0.30		0	0	0.07
Mdh-A		2	1	1	1.41	1.41	2	-	1	1	
		0.30	0	0	0.15	0.15	0.30		0	0	0.11
6Pgd		1.41	1.41	1.41	1.41	2.83	2.83	-		2	
		0.15	0.15	0.15	0.15	0.45	0.45			0.30	0.26
Mean (log)	0.21	0.24	0.21	0.21	0.32	0.25	0.38	-	0.30	0.20	

Mean of all tissues, enzymes = 0.25 (log), 1:1.80 (inverse log)

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