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Genetics

Microcomplement fixation studies on the evolution of α-glycerophosphate dehydrogenase within the genus *Drosophila*

(dipteran evolution/unit evolutionary period)

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ABSTRACT Antisera were prepared against purified α-glycerophosphate dehydrogenase (EC 1.1.1.8) (αGPDH) from *Drosophila melanogaster, D. virilis,* and *D. busckii.* The immunological distances between the enzymes from the 3 species and those from 31 additional drosophilid species agree in general with the accepted phylogeny of the genus. These data permit an estimate that the subgenus *Sophophora* diverged 52 million years ago from the line leading to the subgenus *Drosophila.* The antiserum against *melanogaster* αGPDH was capable of distinguishing allelic variants of αGPDH. On the basis of presumed single amino acid substitutions, no drosophilid αGPDH tested differed from the *melanogaster* enzyme by more than eight or nine substitutions. The study was extended to include representatives of six other dipteran families. The immunological distances between αGPDH from *Drosophila* and αGPDH from these dipterans were reasonably consistent with a phylogeny of the order Diptera established by more conventional means. The unit evolutionary period of this enzyme was estimated to be 18 million years.

Studies on the evolution of proteins have provided biologists with a powerful tool for constructing phylogenies, particularly of taxa that are poorly represented in the fossil record. In general, the phylogenies derived from comparison of protein sequences have agreed well with phylogenies derived by more classical techniques (1–4). Since the insects have a fragmentary fossil record, our understanding of their evolution would greatly benefit from studies of their molecular evolution. Unfortunately, the insects have been grossly underrepresented in studies of molecular evolution (4).

The genus *Drosophila* should be the initial focus of such studies among the insects. It has the best understood phylogeny of any comparably large assemblage of species (5, 6) and its species are the easiest of the higher eukaryotes to manipulate genetically. Agreement between a phylogeny derived from molecular data and the generally accepted phylogeny provided by Throckmorton (6) would provide a basis of confidence for molecular studies applied to related insect groups whose phylogenetic relationships are poorly understood.

The choice of a protein for such studies cannot be casual. Three criteria must be met. First, the protein should occur in most insects. Second, because the study of structure-function relationships in proteins can hardly be separated from studies of their molecular evolution, the protein should be significant functionally. The dimeric enzyme α-glycerophosphate dehydrogenase (EC 1.1.1.8) (αGPDH) meets these criteria. Its role in intermediary metabolism ensures its presence in the tissues of most or all insects. Furthermore, this enzyme is essential, at least in *D. melanogaster,* for rapid production of the energy needed for flight (7–9).

The third criterion is that the protein should be evolving relatively slowly. Although cytogenetic analysis and interspecific hybridization are adequate for establishing phylogenetic relationships among closely related species, a protein that has changed slowly is particularly useful for establishing the relationships among species groups, subgenera, genera, and even families or orders. Brosemer *et al.* (10) and Fink *et al.* (11) have established with immunological tests that the structure of αGPDH has been substantially conserved during the evolution of the Hymenoptera. The results reported here show similar conservatism within the genus *Drosophila* and among other Diptera.

This paper deals with the divergence of αGPDH within the genus *Drosophila* and several related genera as measured by the immunological technique of microcomplement fixation (12). Differences between homologous proteins measured by this technique are directly related to percentage sequence divergence (13, 14). The technique is sensitive enough to detect single amino acid differences (15, 16).

MATERIALS AND METHODS

Antiserum Preparation. αGPDH was purified to homogeneity from *D. melanogaster, D. virilis,* and *D. busckii* by the procedure of Collier *et al.* (17). Four New Zealand white male rabbits were each injected with a total of 100 μg of the purified *D. melanogaster* enzyme. A total of 45 μg of purified *D. virilis* enzyme was injected into each of four additional rabbits. Three rabbits each received a total of 15 μg of purified enzyme from *D. busckii.* The antigen for each rabbit was divided into four 1-ml portions that were administered at weekly intervals. The rabbits were bled from the ear 1 week after the last injection. The antisera exhibited a single precipitin line containing αGPDH activity in double diffusion and immunolectrophoretic analyses.

Antigen Preparation. Each antigen used for microcomplement fixation was partially purified as follows. Adult flies (2 g) were homogenized in 0.1 M sodium phosphate buffer, pH 7.1, containing 10^{-6} M NAD, 0.5 mM dithiothreitol, 1 mM EDTA, and 10 mM α-glycerophosphate. The extract was centrifuged at 15,000 $\times$ g for 20 min and passed over carboxymethyl cellulose and then DEAE-cellulose, each packed in small sintered glass funnels and equilibrated with the homogenization buffer. Under these conditions, αGPDH is not retained on either ion exchange resin, but the eye pigments and many other proteins are retained. About 20-fold purification for each an-

Abbreviations: αGPDH, α-glycerophosphate dehydrogenase; ID, immunological distance.

Table 1. Immunological distances between αGPDH from *D. melanogaster*, *D. busckii*, and *D. virilis* and αGPDH from various drosophilid species

Antiserum*			Antigen†	
mel. A/A	*vir.*	*busckii*	Species	Species group
0	2	0	*melanogaster A/A*	
12	0	8	*virilis*	
6	2	0	*busckii*	
9	—	—	*melanogaster B/B*	melanogaster
5	2	0	*simulans*	melanogaster
6	1	0	*yakuba*	melanogaster
11	—	—	*birchii A/A*	melanogaster
17	—	—	*birchii B/B*	melanogaster
23	8	3	*mimetica*	melanogaster
13	—	—	*affinis A/A*	obscura
19	—	—	*affinis B/B*	obscura
17	—	—	*affinis C/C*	obscura
15	12	7	*algonquin*	obscura
12	4	6	*pseudoobscura*	obscura
16	—	—	*miranda*	obscura
29	10	6	*willistoni*	willistoni
11	7	1	*paulistorum*	willistoni
26	4	6	*emarginata*	saltans
19	2	17	*sturtevanti*	saltans
17	6	14	*Chymomyza procnemis*‡	
27	7	—	*Chymomyza amoena*‡	
25	5	9	*funebris*	
16	0	8	*gibberosa*	annulimana
21	0	8	*montana*	virilis
21	0	13	*micromelanica*	melanica
20	—	—	*paramelanica A/A*	melanica
25	—	—	*paramelanica B/B*	melanica
23	3	13	*robusta A/A*	robusta
28	—	—	*robusta B/B*	robusta
26	0	13	*mercatorum*	repleta
21	3	8	*hydei*	repleta
26	5	11	*immigrans A/A*	immigrans
30	—	—	*immigrans B/B*	immigrans
16	—	—	*nasuta A/A*	immigrans
12	—	—	*nasuta B/B*	immigrans
23	11	13	*Zaprionus vittiger*‡	
13	0	4	*quinaria*	quinaria
19	1	8	*putrida*	testaceae
20	0	0	*cardini*	cardini
17	0	11	*pallidipennis*	pallidipennis
26	8	19	*tripunctata*	tripunctata
19	6	5	*duncani*	
41	28	28	Number of stocks tested	

* A dash indicates that an ID was not determined. *mel.* = *melanogaster; vir.* = *virilis*.

† The capital letters following some species stand for allozymes of αGPDH found in these species.

‡ Despite the nomenclatural distinction given these species, phylogenetically they are a part of the genus *Drosophila* (5, 6).

tigen was achieved. Antigens prepared by this procedure gave microcomplement fixation curves identical to those obtained with pure antigen.

Microcomplement Fixation. Microcomplement fixation experiments were performed and immunological distances (ID) were calculated according to Champion *et al.* (12). The titers of the pooled antisera were 3350 for *melanogaster* enzyme, 2600 for *virilis* enzyme, and 1200 for *busckii* enzyme. Each ID is the average of four determinations. Standard errors were ±10% or less.

RESULTS AND DISCUSSION

Immunological Distances Between Drosophilid αGPDH. Table 1 shows IDs for *Drosophila* species arranged according to the generally recognized species groups. These data show that the three antisera differ in specificity. There is a lack of reciprocity of IDs among *D. melanogaster*, *D. virilis*, and *D. busckii*, perhaps due to errors associated with small IDs. However, because repeated determinations of these values indicate an experimental error of only ±10%, the lack of reciprocity could well be due to the different amounts of antigen used to produce the antisera. In other words, the small amounts of enzymes from *D. virilis* and *D. busckii* used to produce antisera may not have been sufficient to elicit diverse families of antibodies capable of recognizing all the antigenic determinants on the enzyme surfaces. Thus, in addition to having low titers, these antisera would be less discriminating than the antiserum against *melanogaster* enzyme. The remaining values in Table 1 show that the antiserum against *melanogaster* enzyme is in fact the most discriminating of the three. In any case, because the data are not used to construct phylogenetic trees but only to *estimate* degree of change in the enzyme during the evolution of the genus *Drosophila*, the lack of reciprocity among antisera is not critical.

Fig. 1, modified from Throckmorton (6), diagrams the relationships of the species used in this study. These species represent most of the major subdivisions of the genus. The IDs between the species' enzymes in Table 1 conform well with their phylogenetic relationships presented in Fig. 1. In general, with all three sera the smallest IDs are between enzymes from closely related species. This generalization has two major exceptions. First, *D. mimetica* is a member of the *D. melanogaster* species group but immunologically, with each antiserum, its enzyme is as different from the *D. melanogaster* enzyme as are the enzymes from species of other subgenera. The second exception is *D. busckii*. Taxonomists have placed this species in the subgenus *Dorsilopha*. However, as seen in Table 1, the enzyme from *busckii* differs very little from that of *melanogaster*, a member of the subgenus *Sophophora*. The basis of these anomalies may be differences in the rate of evolution of αGPDH in the lines of descent leading to *D. mimetica* and *D. busckii*.

There are other instances of ID heterogeneity within species groups. Until sequence data are available, it cannot be determined if this heterogeneity is due to rate differences or is a consequence of the approximate nature of the immunological measure. Therefore, the phylogenetic relationships revealed by the data in Table 1 are more obvious when the average IDs of species groups are compared (Table 2).

Dating the *Sophophora* Radiation. Only two drosophilid fossils have been found (18, 19). These fossils indicate the Sophophoran radiation to be at least 25–30 million years old and the family must be at least 50 million years old. Throckmorton (6) has argued that the divergence of the subgenera *Sophophora* and *Hirtodrosophila* and the two major radiations of the subgenus *Drosophila* must have occurred by the end of the Eocene (36 million years ago) in order to account for the current species distributions.

The average IDs to the two radiations of the subgenus *Drosophila* (*virilis-repleta* and *immigrans-tripunctata*) are roughly equal when measured with the antiserum against *melanogaster* enzyme (Table 2). This result is consistent with a relatively constant average rate of αGPDH evolution in the genus because

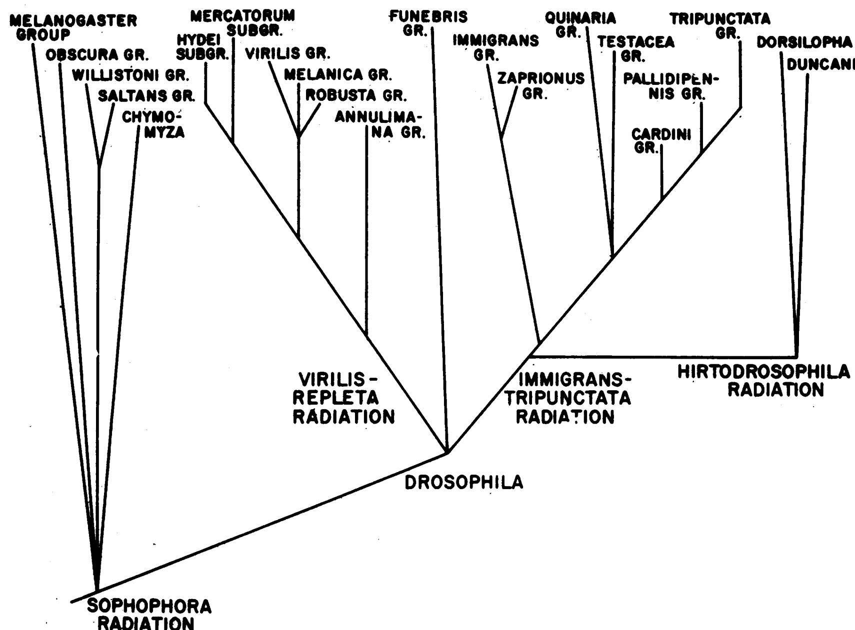

FIG. 1. Diagrammatic representation of the phylogeny of the genus *Drosophila* [adapted from Throckmorton (6)].

both of these major subdivisions have been isolated for the same length of time from *D. melanogaster*. Also, the IDs measured with the antiserum against *virilis* enzyme reflect the fact that *D. virilis*, a member of the *virilis-repleta* radiation, has more recently shared a common ancestor with the species from the *immigrans-tripunctata* radiation (average ID = 3.3) than with species from the subgenus *Sophophora* (average ID = 4.8). Assuming an equal average rate of change of αGPDH in the different lineages and given that the *virilis-repleta* and *immigrans-tripunctata* radiations had diverged from each other by 36 million years ago, the divergence of *Sophophora* from *Drosophila* must have occurred approximately 52 million years ago $(4.8/3.3) \times 36 \times 10^6$.

Sequence Divergence versus Immunologic Distance. Because primary sequences of αGPDH from *Drosophila* species have not yet been determined, ID measurements cannot be directly calibrated in terms of sequence differences. However, naturally occurring allelic electrophoretic variants (allozymes) of this enzyme, available in some species, provide a basis for inferring amino acid substitutions. In Table 1, when more than one allozyme is available for study from a given species, the stock monomorphic for the most common allozyme is designated A/A and stocks monomorphic for the other allozymes are designated B/B or C/C. It is reasonable to assume that the protein produced by the common allele and the proteins produced by the rare alleles in each of these species differ by single amino acids. This assumption is supported by the observations that many of the rare forms of hemoglobin were initially detected as electrophoretic variants, with subsequent sequence studies revealing that the vast majority differ from the corresponding normal globin chain by single amino acid substitutions (20).

Because the antiserum against *melanogaster* enzyme A/A was the most discriminating, it was used to measure the differences in ID between the various allozymic forms of αGPDH. These ID differences, abstracted from Table 1, are presented

Table 2. Average immunological distances to specific groups and subgenera

Antiserum					Antiserum		
mel. A/A	*virilis*	*buschkii*	Species group	Subgenus	*mel. A/A*	*virilis*	*buschkii*
12(8)*	4	1	*melanogaster*				
15	3	6	*obscura*				
15	9	4	*willistoni*	*Sophophora*	15.5	4.8	4.6
23	3	11	*saltans*				
22	6	14	*Chymomyza*				
21	1	10	*virilis* and related groups	*Drosophila*	21.3	0.7	10.1
23	2	11	*repleta*				
21	8	12	*immigrans* and *Zaprionus*		20.2	3.3	9.4
16	2	7	*quinaria, tripunctata*, and related groups	*Drosophila*			
19	6	5	*duncani*	*Hirtodrosophila*			

* In parentheses, average ID excluding *D. mimetica*.

Table 3. Immunological distance differences for allozymes of αGPDH

Species		ΔID
melanogaster	A–B	9
birchii	A–B	6
affinis	A–B	6
affinis	A–C	4
paramelanica	A–B	5
immigrans	A–B	4
nasuta	A–B	4
robusta	A–B	5
Mean		5.4
Mean excluding *D. melanogaster*		4.9

in Table 3. In every case, immunological differences could be detected between allozymes from the same species. Furthermore, excepting *D. melanogaster*, the ID differences are rather uniform, ranging from 4 to 6 with an average of 4.9. The clustering of these ID differences is especially significant in that they occur in species closely related to *melanogaster* (*birchii* and *affinis*) and also in distantly related species (*paramelanica, immigrans, nasuta,* and *robusta*). These observations suggest that the antiserum against *melanogaster* enzyme A/A detects single amino acid substitutions and that each amino acid substitution in the heterologous antigens increases the ID by 4 to 6 units.

The ID difference between the *melanogaster* allozymes is approximately twice as large as the average of the seven other values. This 2-fold difference suggests that the *melanogaster* allozymes may differ by two amino acid substitutions. In this context it should be noted that these allozymes are polymorphic in *D. melanogaster* populations whereas variant allozymes in other species of *Drosophila* are rare and have lower catalytic efficiencies than do their common counterparts (unpublished data). Furthermore, population surveys (21, 22) and biochemical studies (23) indicate that in *D. melanogaster* the allozymes represent an adaptive polymorphism. Plausibly, then, a two-amino acid substitutional difference may have occurred through the fixation of a slightly deleterious amino acid substitution followed by a substitution establishing a new selectively favored amino acid sequence (24, 25).

Fig. 2 includes the frequencies of all of the IDs measured with the antiserum against *melanogaster* enzyme A/A. As expected, the allozyme differences exhibit the lowest ID values. The IDs to the enzymes of the closely related species *D. simulans* and *D. yakuba* are also small. Conspicuous clusters of ID values occur at 12, 16, and 20 units. Although IDs provide only approximate estimates of sequence divergence, these tight clustering of values may represent differences of three, four, and five amino acid substitutions, respectively. If this is the case, none of the drosophilid enzymes differs from the *D. melanogaster* enzyme by more than eight or nine amino acid substitutions.

Unit Evolutionary Period of αGPDH. Because of the conservatism of αGPDH among the drosophilids, the study was extended to include species from other families of Diptera. The IDs for αGPDH from these species, as measured with each of the three *Drosophila* antisera, are listed in Table 4. The species are listed in a proposed order of decreasing phylogenetic distance from *Drosophila* (26). The families Muscidae and Sarcophagidae probably diverged from one another after diverging from the main line of dipteran evolution. Although the antiserum against *melanogaster* enzyme A/A is again the most

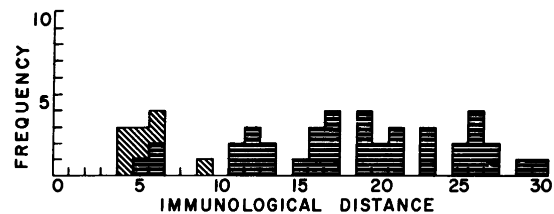

FIG. 2. Histogram of the frequencies of immunological distances measured with the antiserum against *melanogaster* enzyme A/A. The diagonal cross-hatching indicates the immunological distance differences for the allozymes listed in Table 3. The immunological distance values listed in Table 1 for the antiserum against *melanogaster* enzyme A/A are indicated by horizontal cross-hatching.

discriminating, measurements with all three antisera provide the same relative order of IDs. The only discrepancy between the order of IDs and the proposed phylogenetic order is the placement of the family Tephritidae relative to the families Muscidae and Sarcophagidae. If Rhodendorf's (26) proposed order of divergence of these families is correct and the species examined are representative of their families, then the rate of αGPDH evolution has been slightly faster among the tephritids than among either the muscids or sarcophagids. In view of the importance of this enzyme to flight metabolism, it is worth noting that the tephritids are rather lethargic and reluctant fliers compared to the muscids or sarcophagids. Perhaps selection on the gene products involved in flight metabolism has been relaxed in the tephritid lineage, thus accounting for an apparently more rapid rate of evolution of αGPDH.

With dates provided by Rhodendorf's (26) phylogeny, and the approximate number of amino acid replacements estimated from the complement fixation data, we have obtained a preliminary estimate of the unit evolutionary period for αGPDH. This period is the time required for 1% sequence divergence to occur between homologous proteins in different evolutionary lineages (1). The calculations are presented in Table 5. Even if the antiserum does not detect certain amino acid substitutions, and allowing for errors of tens of millions of years for the divergence dates, clearly this enzyme is evolving more slowly than hemoglobin [unit evolutionary period = 5.8×10^6 years (1)]. Indeed, if the errors are small, then at least among the Diptera, αGPDH is evolving almost as slowly as cytochrome *c* [unit evolutionary period = 20×10^6 years (1)].

In general, our results conform well with the proposed

Table 4. Immunological distances between αGPDH from *Drosophila* and αGPDH from other families of Diptera

Antiserum				
mel. A/A	*vir.*	*bus.*	Species	Family
138	93	68	*Megaselia scalaris*	Phoridae
58	45	36	*Cochliomyia hominivorax* (screwworm fly)	Sarcophagidae
64	48	45	*Musca domestica* (house fly)	Muscidae
81	66	44	*Rhagoletis pomanella* (apple maggot)	Tephritidae
74	61	42	*Anastrepha suspensa* (Caribbean fruit fly)	Tephritidae
51	34	28	*Liriomyza sativae* (vegetable leaf miner)	Agromyzidae
48	25	24	*Scatella stagnalis*	Ephydridae

Table 5. Calculation of the unit evolutionary period (UEP) of αGPDH

Branch point of lineage to *D. melanogaster*	Time since common ancestor (year × 10^{-6})	ID	Amino acid substitutions: Approx. no.	Amino acid substitutions: No. per 100 residues*	Amino acid substitutions: Corrected no.†	UEP (year × 10^{-6})
Phoridae	125	138	35	11.7	12.4	10.1
Sarcophagidae	100	58	15	5.0	5.1	19.6
Muscidae	100	64	17	5.7	5.9	16.9
Tephritidae (*R. pomanella*)	90	81	20	6.7	6.9	13
Tephritidae (*A. suspensa*)	90	74	18	6.0	6.2	14.5
Agromyzidae	70	51	13	4.3	4.4	15.9
Ephydridae	65	48	12	4.0	4.1	15.9
virilis-repleta radiation	50	21	5	1.7	1.7	29.4
immigrans-tripunctata radiation	50	20	5	1.7	1.7	29.4
					Mean	18.3

* The subunit molecular weight of this polypeptide (32,250) (17) indicates that it contains about 300 residues (average amino acid molecular weight = 110).

† Corrected for multiple changes at the same position by the formula $m/100 = \ln [1 - (n/100)]$ in which n is the number of observed amino acid substitutions and m is the corrected number (1).

phylogenies of both the genus *Drosophila* and the order Diptera. Sequence studies of this enzyme will therefore provide valuable information with which to interpret the phylogeny of *Drosophila* and other dipterans. Indeed, if αGPDH has evolved as slowly among other insects, with the appropriate tree building techniques (27–30), studies of this enzyme may provide important insights into the phylogeny of the class Insecta.

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