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Nucleotide Sequence Comparison of the Adh Gene in Three Drosophilids

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Summary. The alcohol dehydrogenase (Adh) gene has been isolated from *Drosophila simulans* and *D. mauritiana* by screening λ clone libraries of each with a previously cloned Adh gene from *D. melanogaster*. The isolated λ clones were subcloned and partially sequenced to determine the relatedness of these species and to examine details of evolutionary change in the structure of the Adh gene. We report the sequence of the first 704 nucleotides of each gene as well as 127 bases in the 5′ untranslated region. When these sequences are compared, *D. melanogaster* differs from *D. simulans* and *D. mauritiana* by 2.8% and 3.1%, respectively. *D. simulans* and *D. mauritiana* differ by only 1.8%, implying that they are more closely related to each other than either is to *D. melanogaster*. This is consistent with phylogenetic relationships established by a variety of genetic, biochemical, and morphological means and illustrates that DNA sequencing of a single gene may be used to assess the evolutionary relationships of species.

Key words: Alcohol dehydrogenase — DNA sequence — Drosophilids

Introduction

The alcohol dehydrogenase (Adh) gene of *D. melanogaster* has been intensively studied and has many interesting features. Alcohol dehydrogenase protein (ADH) is abundant and has been purified and characterized (Sofer and Ursprung 1968); its complete amino acid sequence has been determined (Thatcher 1980). In addition, the gene has been cloned and the nucleotide sequence of introns, exons, and flanking DNA determined (Benyajati et al. 1980, 1981; Goldberg 1980; C. Benyajati unpublished). The Adh gene has been genetically and cytologically mapped with considerable accuracy owing to the availability of both overproducing and null mutants (e.g., O'Donnell et al. 1978; Woodruff and Ashburner 1979). Expression of the Adh gene is developmentally regulated and tissue specific (e.g., Ursprung et al. 1970). It has recently been shown that this expression is controlled by two promoters, one of which is active in early development and the other of which functions at later stages (Benyajati et al. 1983).

Our laboratory has been interested in the evolution of the Adh gene. *Drosophila* ADH is quite different from those of mammalian liver and yeast. The subunit molecular weight is less (25,000 daltons in *Drosophila*, 39,800 daltons in liver, and 35,300 daltons in yeast) and *Drosophila* ADH does not require Zn^{2+} for enzymatic activity, as does ADH of other species (Benyajati et al. 1980). Moreover, the amino acid sequence of *Drosophila* ADH shows little homology to that of ADH from liver or yeast. Nevertheless, it has been possible to align the amino acid sequence of the three ADH proteins using computer predictions of secondary structure (Benyajati et al. 1980). Based on this analysis, the positions of two introns in the *D. melanogaster* Adh gene (after amino acids 33 and 168, numbering ATG as codon 1) were examined relative to structural "domains" of the protein. Interestingly, one intron appears to fall between two domains, as predicted by the gene-shuffling hypothesis of Gilbert (1978), while the other does not. It seems worthwhile to examine Adh in other drosophilids to see whether the position of one or both of these introns has been conserved during evolution.

We have previously used a cloned fragment of

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the *D. melanogaster* Adh gene as a probe to quantitate divergence of various species of *Drosophila* by DNA hybridization and thermal elution from hydroxyapatite (Zwiebel et al. 1982). In the present article, we extend these studies by reporting the DNA sequences of the first 704 nucleotides of the Adh gene from *D. simulans* and *D. mauritiana,* as well as of 127 nucleotides in the 5′ untranslated region.

Materials and Methods

Isolation of Adh-Containing λ Clones. Clone libraries of *D. simulans* and *D. mauritiana* were kindly provided by Dr. M. Meselson. These were screened by modification of the procedures of Benton and Davis (1977) as described previously (Zwiebel et al. 1982). Libraries were screened with a *D. melanogaster* Adh-containing clone kindly provided by D. Goldberg. Isolated λ clones were propagated, and DNA was extracted as described by Blattner et al. (1977). Maps of restriction enzyme cleavage sites were constructed by digestion, electrophoresis, and Southern transfer as described previously (Zwiebel et al. 1982).

Subcloning. The isolated λ clones were digested with Eco RI. DNA isolated from the plasmid pBR 325 (chosen because it contains a selectable marker interrupted by an Eco RI site) was digested with Eco RI and treated with calf intestinal alkaline phosphatase (Boehringer Mannheim). The enzyme was suspended in 10 mM Tris–HCl, pH 8.0, 50 mM KCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 50% glycerol; added to Eco RI-digested vector DNA in 10 mM Tris–HCl, pH 8.0; and incubated for 1 h at 65°C. Phosphatased vector DNA was extracted twice with Tris-saturated phenol and once with Sevag (24:1, chloroform/isoamyl alcohol). Vector and λ clone DNAs were combined and coprecipitated. The pellet was resuspended in 5 mM Tris–HCl, pH 7.6; and ligated in 0.6 mg/ml ATP, 66 mM Tris–HCl, pH 7.6, 6.6 mM $MgCl_2$, 10 mM dithiothreitol, and 4.5 units of T4 ligase (BRL) for 25 h at 15°C. Ligated samples were used to transform *E. coli* strain HB101 and cells were plated on L-broth agar plates containing 25 μg/ml ampicillin. Colonies were replica plated onto chloramphenicol-containing plates, and those which failed to grow were cultured and screened by Southern Transfer for the presence of Adh-containing fragments. Positive subclones were grown and their DNA was isolated; maps of restriction enzyme cleavage sites were constructed as described above.

DNA Sequencing. DNA sequencing was performed by the chemical cleavage method of Maxam and Gilbert (1977). Three types of ^{32}P end-labeling were employed: (a) labeling at 3′ recessed ends with the large fragment of polymerase I (BRL); (b) labelling at 5′ protruding ends with polynucleotide kinase (P-L Labs); and (c) labeling at 3′ protruding ends with terminal deoxynucleotide transferase (NEN) and cordycepin 5′-triphosphate (α-^{32}P). Chemical reactions, electrophoresis, and gel elutions were performed using standard methods. Confirmation of sequence was obtained by sequencing both strands when possible, by overlapping fragments (see Fig. 1), and by repeated labelings.

Results

The Adh gene was isolated from genomic λ clone libraries of *D. mauritiana* and *D. simulans* by Benton and Davis's (1977) plaque hybridization method using a cloned *D. melanogaster* Adh gene as the probe. Restriction maps were constructed using standard techniques. The λ clones were digested with Eco RI and the resultant fragments subcloned into the plasmid pBR 325. The subclones were extensively mapped for restriction sites, and then regions containing the Adh gene were sequenced using the procedure of Maxam and Gilbert (1977). The sequencing strategy, which employed overlapping fragments and both 3′ and 5′ labeling, is outlined in Fig. 1. Confirmation of sequence was obtained by sequencing both strands whenever possible, and by repeated labelings. The sequences of 831 nucleotides within and 5′ to the Adh genes of *D. simulans* and *D. mauritiana* were determined and are shown in Fig. 2. The sequences include DNA 5′ to the gene, the first two exons, and both introns, as well as a portion of exon 3. The *D. melanogaster* Adh sequence determined by C. Benyajati (1981 and unpublished data) is shown for comparison.

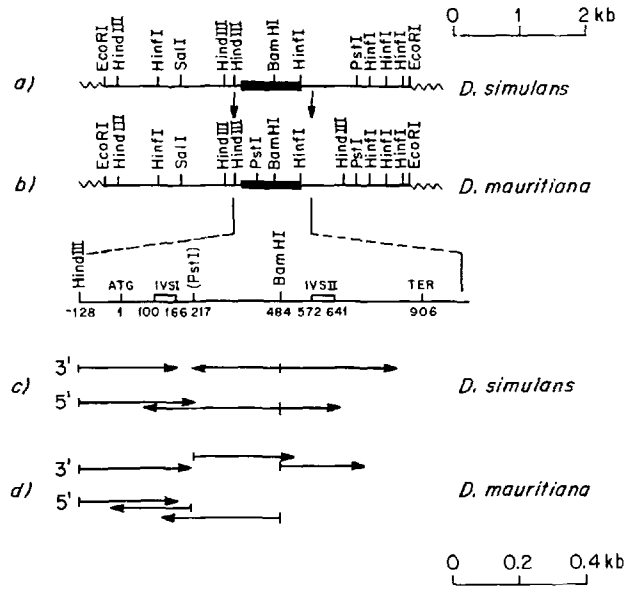

Fig. 1. Restriction enzyme cleavage sites in Adh-containing clones from (a) *D. simulans* and (b) *D. mauritiana.* The Adh gene itself is indicated by a thickened line; the wavy line indicates the vector pBR 325. Strategies used in DNA sequencing of the Adh gene of *D. simulans* and *D. mauritiana* are shown in (c) and (d). The Pst I site in parentheses in the schematic occurs only in *D. mauritiana.* IVSI, Intron 1; IVSII, intron 2; TER, termination of translation

The determined sequence starts 127 nucleotides upstream from the ATG start site of translation. Two deletions/insertions of a single base occur in the 5′ flanking region of the gene. 102 nucleotides upstream from the ATG codon is an AT-rich region (TATAAATA), identical in all three species, that may correspond to the TATA or Goldberg–Hogness box commonly found 5′ to eucaryotic genes (Goldberg 1979). The distance between this region and the ATG site is approximately that expected based

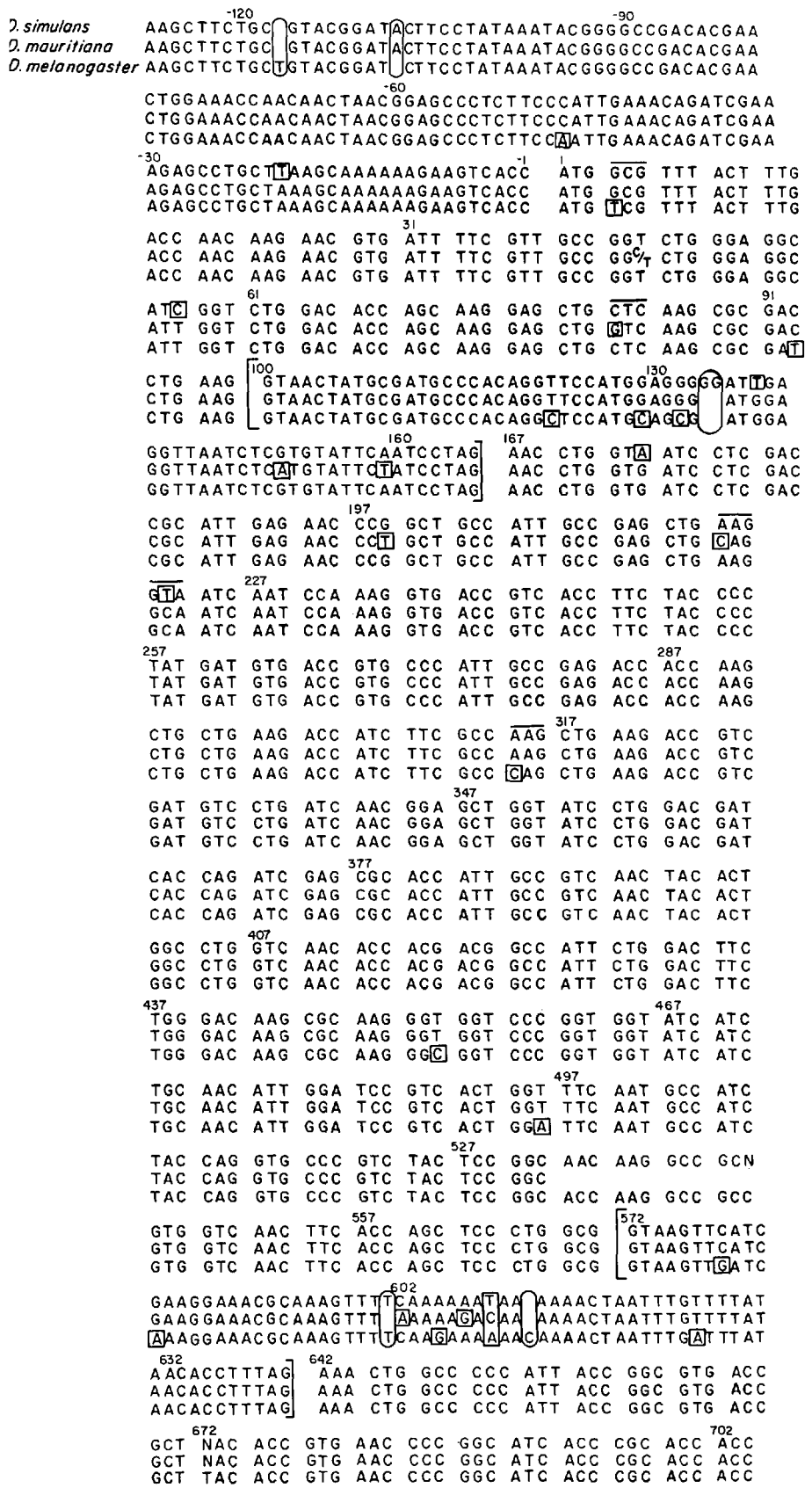

Fig. 2. DNA sequences within and 5′ to the Adh genes of *D. simulans* and *D. mauritiana*. The previously determined sequence of *D. melanogaster* Adh (Benyajati et al. 1981 and unpublished) is shown for comparison. The position of introns is indicated by brackets. Nucleotide substitutions are boxed and deletions/insertions are indicated by circles. Codons that undergo nucleotide change leading to amino acid replacement are overlined. The gap at nucleotide 533 indicates a region of twelve nucleotides that have not been determined in *D. mauritiana*. Sequence ambiguities (positions 45, 544, and 672) have not been scored as nucleotide changes in Tables 1 and 2. Position 1 is the ATG start site of translation

on the estimated length of the 5′ leader sequence of Adh mRNA of adult flies (Benyajati et al. 1980). The eight nucleotide AT-rich region is followed immediately by eight nucleotides of GC. Intron 1 occurs at nucleotide 100 (after amino acid 33) and intron 2 at nucleotide 572 (after amino acid 168) in

Table 1. Percentage of DNA sequence divergence in and around the Adh gene of three drosophilids[a]

Species	5′ flanking DNA	Exons			Introns		All exons	All introns	Entire sequence
		1	2	3[b]	1	2			
D. simulans/D. melanogaster	3.1	3.0	1.2	0	7.5	8.6	1.4	8.0	2.8
D. simulans/D. mauritiana	0.8	2.0	1.0	0	6.0	5.7	1.1	5.8	1.8
D. mauritiana/D. melanogaster	2.4	3.0	1.3	0	7.5	12.9	1.4	10.2	3.1

[a] Deletions/insertions are scored as single events
[b] Calculated based on the first 63 nucleotides of exon 3

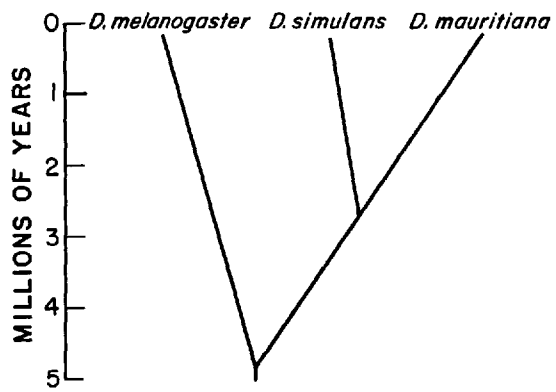

Fig. 3. Evolutionary relationship among three drosophilids as deduced from nucleotide sequence comparison of the Adh genes. The indicated divergence times are maximal estimates

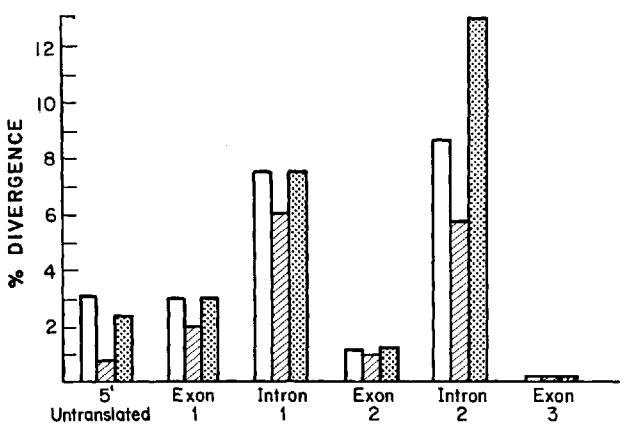

Fig. 4. Distribution of base changes in the Adh genes of three drosophilids. The open bar indicates *D. melanogaster* compared with *D. simulans*; the hatched bar indicates *D. mauritiana/D. simulans*; and the stippled bar indicates *D. melanogaster/D. mauritiana*. The data are taken from Fig. 2 and Table 1. There are no nucleotide changes within the 63 sequenced nucleotides of exon 3

all three species. Both introns conform to the consensus splicing rules suggested by Breathnach and Chambon (1981). The lengths of the introns vary slightly among the three species due to short insertions/deletions, which are common in eucaryotic introns (Moore 1983). The introns are more AT rich than is the structural gene; this is particularly true for intron 2, which is approximately 74% AT.

Over the entire length of the determined sequence, *D. simulans* and *D. mauritiana* are clearly more similar to each other than either is to *D. melanogaster*. As shown in Table 1, *D. simulans* and *D. mauritiana* differ from each other in 1.8% of the bases, whereas they differ from *D. melanogaster* by 2.8% and 3.1% of the bases, respectively. This sequence comparison suggests the evolutionary relationship among the three species outlined in Fig. 3, which indicates that *D. simulans* and *D. mauritiana* diverged from each other after their joint divergence from *D. melanogaster*. This phylogeny agrees with others constructed on the basis of both molecular and nonmolecular characteristics (see Discussion).

DNA sequence divergences can be compared in 5′ flanking DNA, each exon, and each intron (see Table 1 and Fig. 4). The introns diverge at least five times more rapidly than do the coding regions of the gene. Intron 2 may diverge slightly more rapidly than intron 1, and exon 1 may diverge slightly more rapidly than exon 2, but the differences are not statistically significant. Although there is little divergence in exon 3, the sequence has not been determined completely; thus a full comparison cannot yet be made. The 5′ flanking DNA diverges significantly less rapidly than does DNA in introns, which may be the result of evolutionary constraints on sequence divergence in this region.

Sequence divergence can also be examined with regard to placement of individual nucleotide changes, as shown in Table 2. Nucleotide changes in the third positions of codons are somewhat more frequent than changes in position 1. Changes in position 1 are four times as common as those in position 2. Nucleotide changes that lead to amino acid substitutions are less frequent than silent changes, but this effect is not as strong as might have been expected. When the three sequences are aligned, there are 12 silent and 10 replacement changes. The ratio of silent to replacement changes increases significantly, however, when the data are corrected using the method of Perler et al. (1980). Using this method to correct for the potential number of silent and

Table 2. Number of nucleotide differences among the Adh genes of three drosophilids

Species compared	Change in codon position[a]			Silent changes[a]	Amino acid replacement changes[a]	Insertions or deletions[b]	Transitions[b]	Transversions[b]
	1	2	3					
D. simulans/D. melanogaster	2	1	5	5	3	4	8	11
D. simulans/D. mauritiana	2	1	3	3	3	2	7	6
D. mauritiana/D. melanogaster	4	0	4	4	4	4	8	13

[a] Based on comparison of coding DNA only; data from Fig. 2
[b] Based on comparison of introns, exons, and 5′ flanking DNA; data from Fig. 2

replacement sites in each codon, the ratio of silent to replacement changes increases to 5.7 for *D. simulans/D. melanogaster,* 2.7 for *D. simulans/D. mauritiana,* and 3.4 for *D. melanogaster/D. mauritiana.* There does not appear to be any special bias toward transitions rather than transversions, such as has been reported for mitochondrial DNAs (e.g., Brown et al. 1982).

Insertion or deletion events in noncoding DNA have occurred often since the species diverged. It is difficult to distinguish conclusively between insertions and deletions. However, assuming that the phylogeny of Fig. 3 is correct (see Discussion), those cases in which *D. melanogaster* shares a nucleotide(s) with one, but not both, of the other two species are probably the result of an event that took place in the species with the unique base(s). For example, in the first intron, at nucleotide 134, there are two nucleotides that occur in *D. simulans* but not in *D. melanogaster* or *D. mauritiana.* It seems most likely that an insertion has occurred in *D. simulans* rather than two independent deletions at the same location in *D. melanogaster* and *D. mauritiana.* In coding DNA there is no evidence of clustering of base changes, which would indicate "hotspots" of mutational events; there is no single position at which each species has a different base. In noncoding regions, base changes and insertions/deletions do appear to be clustered. The ratio of insertions/deletions to base changes in noncoding DNA is 0.32; this is similar to the value of 0.28 reported by Cann and Wilson (1983) for many noncoding DNAs.

Discussion

The DNA sequence of the Adh gene has been compared among three species of *Drosophila.* Although many genes have been compared in related eucaryotes, very few have been compared among drosophilids. The organization of the genome as a whole, as well as of individual genes, in *D. melanogaster* is different from that of most other eucaryotes in important features such as genome size, interspersion of repeats, intron size and number, etc. (see Spradling and Rubin 1981 for a review). To understand the unusual evolutionary history of drosophilids, detailed interspecies comparisons at the DNA level will have to be made.

Adh is an excellent gene with which to study evolution in drosophilids at the DNA level. It is a small gene and occurs in all species examined. Moreover, an enormous amount of information on it exists with which to put evolutionary comparisons into context. Adh has been analyzed extensively with respect to protein chemistry, genetics, cytology, development, geographical distribution of alleles, and, more recently, the structure of the gene itself. Interspecies comparison may suggest what features of gene structure are critical (thus evolutionarily conserved) and may reveal the nature and rate of evolutionary change.

In this initial study, we have chosen to investigate three species known by several criteria (see below) to be closely related. The advantage in comparing closely related species is that observed differences probably reflect primary events unlikely to be obscured by secondary mutations. Clearly it will also be important to extend this comparison to more distantly related species. The kinds of evolutionary changes observed in the *Drosophila* Adh gene seem analogous to those of gene evolution in other species. For example, the introns diverge more rapidly than the exons; intron position is conserved, but intron length varies as the result of short insertions or deletions; introns are more AT rich than the rest of the gene; and intron/exon borders conform to the consensus sequences seen in other species. Intron position is conserved even in the case of an intron that does not fall between obvious domains of the protein. Conservation of an intron that interrupts a domain may be interpreted as an exception to Gilbert's (1978) suggestion of intron shuffling; however,

there may be domains of the Adh protein of which we are unaware. Intron/exon junctions have been shown to occur outside the hydrophobic core, on the surface of the corresponding protein (Craik et al. 1982). Gō (1981) has proposed that introns fall between subdomains or "modules." The first intron of the Adh gene may separate such modules. Certainly analysis of more distantly related species of *Drosophila* will be important in this context.

It is of particular interest to compare the data presented here with those of Kreitman (1983), who reported the sequences of 11 cloned Adh genes from five natural populations of *D. melanogaster*. Kreitman identified 14 polymorphic sites in coding DNA, only one of which leads to an amino acid replacement. From the low number of amino acid polymorphisms he concluded that virtually all the amino acids of the protein are under selection pressure. The interspecies comparisons reported here show a significantly greater number of amino acid replacements, which suggests that these sibling species may be under somewhat different selective constraints. Ultimately, detailed comparison of interspecies and intraspecies divergence may enhance our understanding of the relationship between speciation and polymorphism.

In addition to demonstrating nucleotide polymorphisms, Kreitman's analysis revealed differences in length (i.e., deletions/insertions) among the alleles. Most of these involved homonucleotide runs. We observed similar mutations among the three species studied here at, for example, positions 134, 601, and 612. Kreitman reported, as we do, that introns diverge more rapidly than exons and that flanking DNA diverges more slowly than introns. Langley et al. (1982) and Kreitman both reported that DNA flanking the Adh gene is as highly conserved as that of the structural gene itself, and concluded that large regions of the genome near the Adh gene may be under selective constraints. Comparison of our data with those of Zwiebel et al. (1982) reveals that the entire single-copy component of the genome diverges only slightly faster than does the Adh structural gene, but more than four times more slowly than do the introns. Taken together, these data suggest that much of the DNA of the *Drosophila* genome may be under sequence-specific selection pressure. Grula et al. (1982), using a very different approach, compared two sea urchin genomes and came to a similar conclusion.

The phylogenetic relationships shown in Fig. 3 have been established on the basis of several criteria, including the pattern of polytene chromosome inversions; protein allelomorphs; interspecies sterilities; mating songs; and molecular similarity in heat-shock genes, mitochondrial DNA, ribosomal RNA, serum proteins, and histone genes (reviewed by Dover et al. 1982). Conclusions from DNA sequence comparisons of the Adh gene reported here are entirely consistent with those of these other studies. This fact is interesting not in that it corroborates previous measurements, but rather because it supports the idea that in at least some cases reliable phylogenies can be constructed on the basis of the DNA sequence of a single gene.

Table 3. Estimated divergence times of three drosophilids based on sequence divergence of the Adh gene

Species compared	Divergence of Adh gene (%)[a]	Estimated time of divergence (millions of years ago)[b]
D. simulans/D. melanogaster	2.8	4.2
D. simulans/D. mauritiana	1.8	2.7
D. mauritiana/D. melanogaster	3.1	4.7

[a] Total divergence including exons, introns, and 127 nucleotides 5′ to the gene; from Table 1

[b] Obtained by division of the percentage divergence of the Adh gene by the minimum rate of divergence of total single-copy DNA in drosophilids (0.66% per million years) calculated by Zwiebel et al. (1982). These are maximal estimates as described in the text

Although the phylogeny of Fig. 3 is well established, it is much more difficult to determine the times at which the species diverged. In a previous study we measured the extent of divergence of total single-copy DNA in a number of drosophilids using DNA hybridization and thermal elution from hydroxylapatite (Zwiebel et al. 1982). Based on the single-copy divergence rate and on divergence times estimated from several unrelated criteria, we estimated the nucleotide substitution rate of drosophilids to be at least 0.66% of bases per million years. This rate can be applied to the extent of divergence of the Adh gene reported here to yield an estimate of the divergence times of these species. Dividing the degree of divergence of the Adh gene by the rate of nucleotide substitution yields estimates of 4.2, 2.7, and 4.7 million years since the separations of *D. simulans/D. melanogaster*, *D. simulans/D. mauritiana*, and *D. mauritiana/D. melanogaster*, respectively (Table 3). Since the nucleotide substitution estimate is a minimal approximation, these times probably represent a maximal estimate.

These estimates of divergence time are highly speculative and based on at least two assumptions. The first is that the Adh gene is diverging at approximately the same rate as total single-copy DNA. This appears to be the case, because the extent of single-copy divergence of *D. melanogaster* with respect to *D. simulans* or *D. mauritiana* was measured to be about 2.2% (Zwiebel et al. 1982), which is similar to the overall degree of divergence of the

Adh gene (Table 3). The second assumption is that single-copy DNA divergence of drosophilids is approximately linear with time. Additional research will be required to test the validity of this concept.

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