

Adaptive evolution at the molecular level of the duplicated *Amy* gene system in *Drosophila*

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Abstract. Analyses of the nucleotide sequences of the duplicated *Amy* genes in the eight species of the *Drosophila melanogaster* species subgroup have revealed concerted evolution of the coding regions and divergent evolution between the duplicated genes of the 5'-flanking regions. Homogenization between the duplicated genes in the coding region is maintained by frequent genetic exchange in various portions of the coding region. On the other hand, such genetic exchange seems to produce a large amount of DNA sequence variation and protein polymorphism at the two loci within a species. The puzzling observation that concerted evolution is restricted to the coding regions seems to be explained by not only adaptive evolution of the AMY proteins in speciation but also adaptive fixation of selectively advantageous mutations in the intergenic region that differentiate expression of the two *Amy* genes. We review molecular work on the *Amy* gene system in *Drosophila*, including evidence from biochemical characterization of the AMY proteins and molecular characterization of the cis regulatory elements.

Keywords. Adaptive evolution; amylase; duplicated genes; *Drosophila*.

1. Introduction

The α -amylase (EC 3.2.1.1, α -1,4-glucan-4-glucanohydrolase) system in *Drosophila* is one of the most extensively studied enzyme systems for an understanding of adaptive evolution. α -Amylase is a digestive enzyme and breaks down starch into glucose and maltose. Thus the enzyme directly interacts with food environments. Indeed, the activity of α -amylase is induced by the substrate (starch induction) and repressed by the product (glucose repression) (Hoorn and Scharloo 1978; Hickey and Benkel 1982; Yamazaki and Matsuo 1984; Matsuo and Yamazaki 1984; Benkel and Hickey 1986a, b; Norman and Doane 1990; Magoulas *et al.* 1993a, b; Inomata *et al.* 1995b). In natural populations of *D. melanogaster*, α -amylase is highly polymorphic in both isozyme frequencies and activities (Abe 1958; Kikkawa 1964; De Jong *et al.* 1972; Hickey 1979; Singh *et al.* 1982; Yamazaki *et al.* 1984; Langley *et al.* 1988). There is also genetic variation in regulatory factors that control the responses to dietary carbohydrates (Hoorn and Scharloo 1978; Hickey and Benkel 1982; Yamazaki and Matsuo 1984; Matsuo and Yamazaki 1984; Benkel and Hickey 1986a, b), tissue specificity (Abraham and Doane 1978; Powell *et al.* 1980; Klarenberg *et al.* 1986), and stage-specific expression patterns (Yamazaki 1986; Da Lage and Cariou 1993). Interestingly, the regulating effect with respect to dietary carbohydrates (inducibility) positively correlated with fitness (Yamazaki and Matsuo 1984; Matsuo and Yamazaki 1984). This indicates that genetic variation of regulatory factors for food environments is raw material for adaptive evolution.

The *Amy* locus has been cloned and well characterized in *D. melanogaster* (*Amy*, 2–80; 54A–B1; Gemmill *et al.* 1985; Levy *et al.* 1985; Gemmill *et al.* 1986; Boer and Hickey 1986; Inomata *et al.* 1995a). The *Amy* structural gene is composed of one exon of 1482 nucleotides. It is predictably translated into a polypeptide with a length of 494 amino acid residues. However, it is predicted that the first 18 amino acids from the N-terminus would be cut off after translation and hence the mature protein would be composed of the remaining 476 amino acid residues (Boer and Hickey 1986).

The *Amy* gene of *D. melanogaster* is duplicated. The two genes are called the proximal and distal genes, and they are in inverted orientation with respect to each other (Gemmill *et al.* 1986; Langley *et al.* 1988). The *Amy* genes of all eight sibling species in the *melanogaster* species subgroup have the same structure as those of *D. melanogaster* (Dainou *et al.* 1987; Payant *et al.* 1988; Shibata and Yamazaki 1995). Therefore the duplication event of the *Amy* genes is considered to predate the diversification of this species subgroup. Multiple copies of the *Amy* gene are also found in other species than the siblings of *D. melanogaster* (Doane *et al.* 1987; Brown *et al.* 1990; Da Lage *et al.* 1992; N. Inomata, H. Tachida and T. Yamazaki, unpublished result). The gene structure in them is different from that in *D. melanogaster*. Thus duplication events of the *Amy* gene seem to have occurred independently in *Drosophila* lineages. Gene duplications followed by diversification of the copies with regard to gene expression and amino acid sequence are considered to be important factors for progressive changes of organisms (Ohno 1970; Ohta 1988). For these reasons the α -amylase system in *Drosophila* is considered to be suitable for the study of adaptive evolution.

Here we review recent molecular work on the duplicated *Amy* gene system in *Drosophila* primarily done in our laboratory. Analyses of sequence variation suggested that positive selection acted on both the coding and the flanking regions of the duplicated *Amy* genes. Concerted evolution, found only in the duplicated coding regions, is maintained by frequent genetic exchange in various portions of the coding region and by natural selection.

2. Evolution of the expression patterns of α -amylase in *Drosophila*

The α -amylase of *Drosophila* shows a variety of expression patterns. Inomata *et al.* (1995b) surveyed the response patterns to dietary carbohydrates of *Amy* gene expression in 20 *Drosophila* species. They showed that α -amylase was repressed by dietary glucose (glucose repression) and induced by dietary starch (starch induction) in many *Drosophila* species, including *D. melanogaster*. In general glucose repression was a well-conserved trait in both larval and adult stages, while starch induction was mainly observed in the larval stage. The degree of response to dietary carbohydrates differs among species. Even among sibling or closely related species there were considerable changes in *Amy* expression pattern, and thus some of the changes of expression pattern were considered to have occurred recently. On the other hand, the same expression patterns were observed among some distantly related species. Therefore the response of α -amylase activity to dietary carbohydrates in *Drosophila* seems to be evolving: glucose repression and larval starch induction have been maintained but their magnitudes have changed to adapt to environments in which the species live.

3. Amount and nature of variation at the molecular level underlying the AMY electromorphs in *D. melanogaster*

In natural populations of *D. melanogaster* α -amylase is highly polymorphic and 12 different electromorphs are detected (Kikkawa 1964; Doane 1969; Hickey 1979; Singh *et al.* 1982; Yamazaki *et al.* 1984; Dainou *et al.* 1987). The adaptive significance of the AMY electromorphs has been discussed in the manner of that of other proteins. Inomata *et al.* (1995a) examined the amount and nature of DNA variation underlying the AMY electromorphs. They analysed 20 sequences of the duplicated *Amy* genes from 10 strains of *D. melanogaster* that possessed different AMY electromorphs. Nucleotide diversity was examined considering the proximal and distal genes separately. There was considerable genetic variation within and between the duplicated genes (within $\pi = 0.012$, between $\pi = 0.011$). Nucleotide diversity within identical electromorphs was also estimated ($\pi = 0.006$). There are many synonymous differences within the AMY1 and the AMY3 electromorphs, and many nonsynonymous differences within the AMY3 electromorph compared with those within the other electromorphs.

Variation of the AMY electromorphs was also estimated at the amino acid level (Inomata *et al.* 1995a). The average number of pairwise amino acid substitutions in natural populations was 1.9. On average there was 1.0 amino acid substitution between identical electromorphs and 3.9 between different electromorphs. Thus, even when two proteins of the same electromorph are randomly chosen, there will be on average 1.0 amino acid substitution between them. To determine the change of relative charge of each electromorph, they were scored +1, -1 or 0 when the amino acid residue at the substituted position was charged positive or negative or was neutral respectively. The scores for AMY1, AMY2, AMY3, AMY4, AMY5 and AMY6 electromorphs turned out to be -3, -2, -1, 0, +1 and +2, respectively. This indicates that the evolution of AMY1 through AMY6 occurred by sequential accumulation of single-charge amino acid substitutions each causing one charge difference.

Inomata *et al.* (1995a) examined the substitution patterns in detail and showed that many genetic exchanges, such as gene conversion or reciprocal recombination, were common both between the duplicated genes and within each locus. To visualize this they sampled the region between sites 921 and 1146 of the coding region and showed each sequence schematically (figure 1). It is clear from the figure that each strain is composed of mosaics of DNA sequences from other strains. If each site has experienced at most one mutation and there has been no recombination, we expect pairs of segregating sites to show a pattern consistent with the nested set of relationships defined by the genealogy of the sample. Observation of all four possible dinucleotide pairs is evidence for recombination between two sites. Three different locations of exchange, indicated by arrows in figure 1, were detected by this method in the region between sites 921 and 1146; namely between 921 and 945, between 945 and 990, and between 999 and 1008. In the region covering the whole coding region of *Amy*, 14 different recombination sites were found in total. This clearly shows that sequence homogeneity between the duplicated genes is maintained by frequent genetic exchange in various portions of the coding region. Genetic exchange is reflected in variation of the AMY electromorphs. One of the AMY3 electromorphs fortuitously has the same mobility as the other AMY3 electromorphs, despite having four amino acid substitutions compared with the latter sequences. Inomata *et al.* (1995a) suggested that gene conversion between the AMY4 and AMY1 or AMY2 genes has occurred around

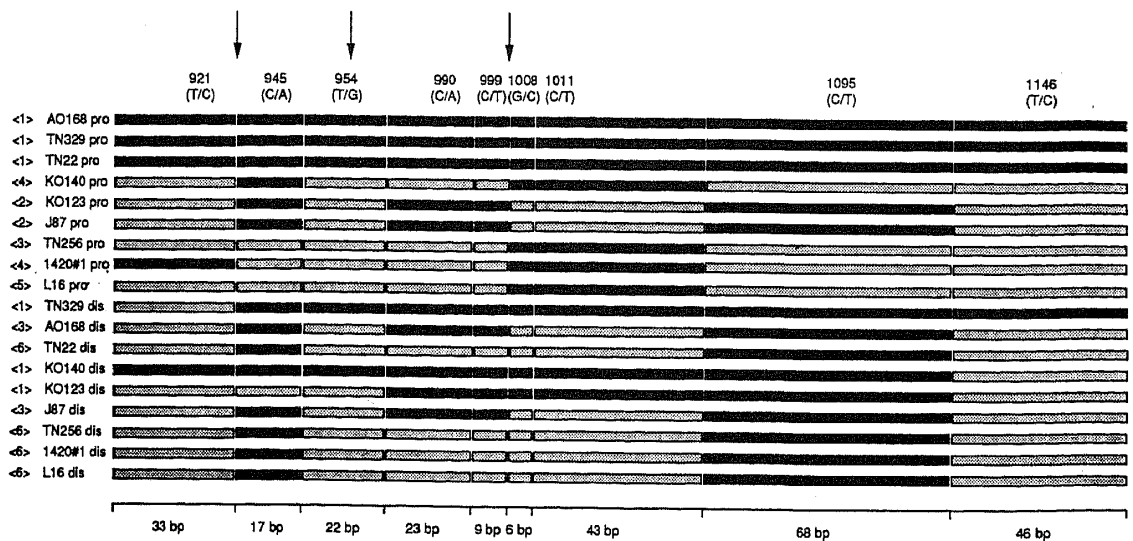


Figure 1. A schematic figure of the nucleotide substitution patterns in the region between sites 921 and 1146 of the *Amy* coding region in nine strains of *D. melanogaster*. Each fragment represents a nucleotide sequence containing a segregating site. Black fragments are sequences identical to the corresponding fragment of the AO168 proximal gene and gray ones indicate a substitution. The boundary between neighbouring fragments is considered to be in the middle of the two segregating sites concerned. Vertical arrows indicate locations of recombination. The leftmost column shows the expressed electromorphs. 'pro' and 'dis' following the strain designation denote proximal gene and distal gene respectively. (From Inomata *et al.* 1995a)

a small fragment including the amino acid substitution with a charge difference and resulted in the two types of AMY3 electromorphs.

4. Molecular structure and nucleotide sequence variation of the *Amy* genes in the *melanogaster* species subgroup

Duplication of the *Amy* genes in the eight siblings of the *D. melanogaster* species subgroup was predicted by genetic analysis and genomic Southern blotting (Dainou *et al.* 1987; Payant *et al.* 1988). Shibata and Yamazaki (1995) cloned and completely sequenced the *Amy* genes of the seven siblings (*D. simulans*, *D. mauritiana*, *D. sechellia*, *D. erecta*, *D. orena*, *D. teissieri* and *D. yakuba*) in addition to those of *D. melanogaster*. They verified that the *Amy* locus of all members of the *melanogaster* species subgroup has an inverted duplication. The nucleotide lengths between the proximal and distal copies were almost the same as that in *D. melanogaster* (~4.5 kb). There was a single exon (1482 bp) without introns in all the sibling species, except for the distal copy of *D. sechellia*. This distal copy had a 35-bp deletion and a 4-bp insertion within the exon. The reading frame was shifted by the deletion and then a termination codon emerged at 30 bp downstream from the deletion. Therefore the distal copy of *D. sechellia* was considered to be a pseudogene. There were 150 substituted sites and eight multiple-substituted sites among the eight sibling species, excluding the deletion and insertion found in *D. sechellia*. On the basis of the coding sequences of the *Amy* gene in the eight species, a phylogenetic NJ tree (neighbour joining method) with the bootstrap probability was constructed (figure 2). It is clearly seen that the proximal and distal genes of the same species are the closest to each other in all species except *D. simulans*. The tree is

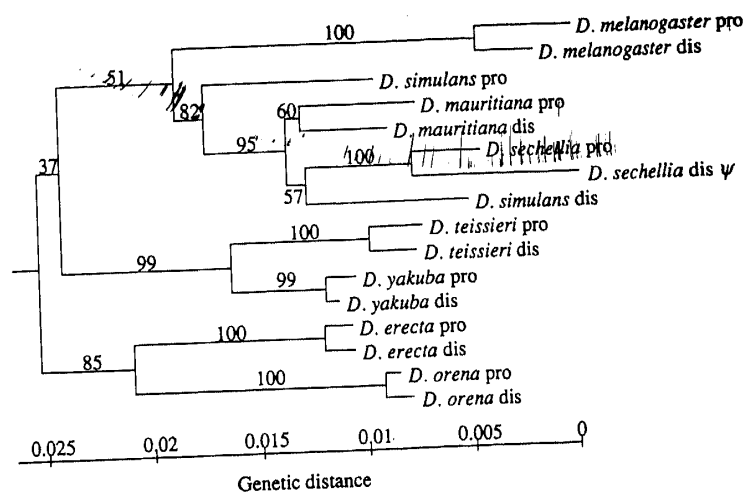


Figure 2. A phylogenetic tree constructed by the neighbour joining method from nucleotide sequence data for the *Amy* coding regions (1482 bp) in *D. melanogaster*, *D. simulans*, *D. mauritiana*, *D. sechellia*, *D. erecta*, *D. orena*, *D. teissieri* and *D. yakuba*. The tree was rooted using the data for *D. pseudoobscura* presented by Brown *et al.* (1990). The numbers above the branches indicate the bootstrap probability (100 trials). pro, Proximal copy; dis, distal copy. (From Shibata and Yamazaki 1995)

striking evidence in support of concerted evolution of the duplicated *Amy* genes, because it is highly unlikely that duplication of the *Amy* locus occurred repeatedly. The phylogeny consists of three large clusters, which match three species complexes, i.e. the *melanogaster* complex, the *yakuba* complex, and the third complex. This cladogram is generally consistent with the phylogenetic trees constructed from various interspecific characteristics of these species (e.g. Eisses *et al.* 1979; Ohnishi *et al.* 1983; Ashburner *et al.* 1984; Solignac *et al.* 1986; Cariou 1987; Jeffs *et al.* 1994).

Shibata and Yamazaki (1995) found differential evolution of the 5'-flanking region of the duplicated *Amy* genes in the *melanogaster* species subgroup. A dot-plot analysis using 4.5 kb of the internal sequence between the proximal and distal *Amy* genes of *D. melanogaster* indicates that an ancestral duplication event of the *Amy* coding region together with 450 bp of the 5'-flanking region has occurred (E. Okuyama and T. Yamazaki, unpublished result). Therefore Okuyama *et al.* (1996) sequenced and analysed 450 bp of the 5'-flanking regions of the duplicated *Amy* genes in the eight species of the *melanogaster* species subgroup. They confirmed that the 5'-flanking sequences were clustered into proximal and distal groups (figure 3). In addition they quantified nucleotide divergence in the 5'-flanking region. A mosaic pattern of highly conserved and divergent regions in the 5'-flanking region of the duplicated *Amy* genes was revealed from the pattern of variation along the sequence (figure 4). Two elements were found in the highly conserved regions between the proximal and distal sequences; the first element, GATAAG (Magoulas *et al.* 1993a), is located at around position -110 and the second one, CCAGTCAATAC/GGTCTGC (Boer and Hickey 1986), is around -230.

5. Analysis of the regulatory sequences in the 5'-flanking region of the duplicated *Amy* genes

Several putative regulatory motifs located in the 5'-flanking region of the *Amy* genes have been reported in *D. melanogaster* (Boer and Hickey 1986; Magoulas *et al.* 1993b). Regulatory sequences required for high levels of expression of one of the duplicated

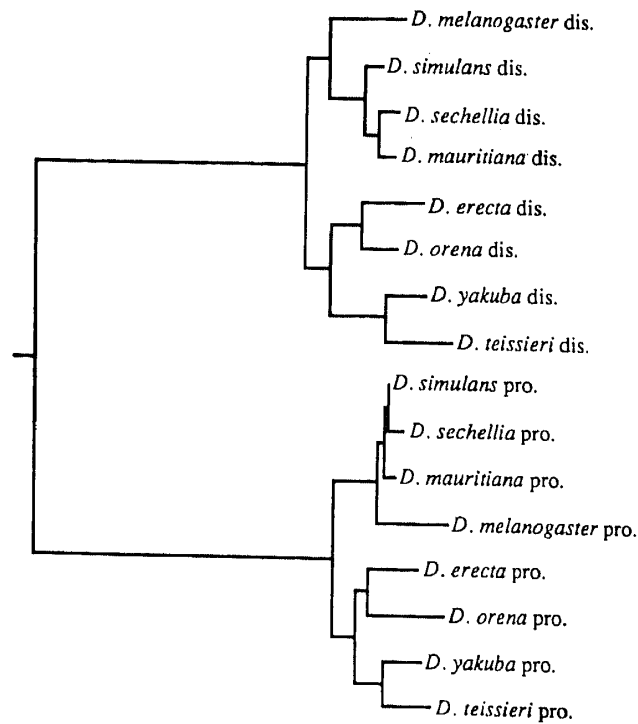


Figure 3. Neighbour-joining tree of the total 5'-flanking region of the *Amy* genes in the eight species of the *D. melanogaster* species subgroup. Roots of the trees are chosen at the centre of the longest route of the tree. The abbreviations are the same as in figure 2. (Modified from Okuyama *et al.* 1996)

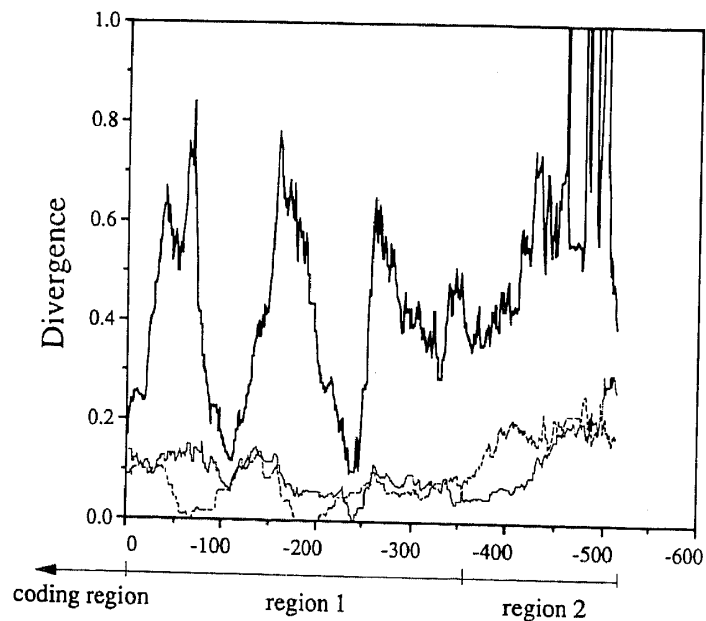


Figure 4. The pattern of divergences within and between the proximal and distal groups (see figure 3 and text) of the 5'-flanking region of the *Amy* genes in the *D. melanogaster* species subgroup. Window size was 50. The thick line represents divergence between the proximal and distal groups. The broken line and dotted line (bottom) represent divergences within the proximal and distal groups respectively. The translation initiation site of the proximal gene of *D. melanogaster* is numbered as reference point 1 with the upstream sites given negative values. The positions of region 1, region 2 and coding region are indicated under the graph. (From Okuyama *et al.* 1996)

Amy genes, the distal gene, in *D. melanogaster* were searched for and identified by Hawley *et al.* (1992) using a transient expression assay in somatic transformants of *D. melanogaster* (Martin *et al.* 1986; Hawley *et al.* 1990). Choi and Yamazaki (1994) examined the intergenic region between the duplicated *Amy* genes of *D. melanogaster* to identify regulatory motifs for full expression of the other *Amy* gene, the proximal gene, by the same technique that Hawley *et al.* (1992) used. They showed that about 70 bp of sequence of the 5' upstream region of the *Amy* distal gene was necessary for full expression of the *Amy* proximal gene. The 70-bp sequence also resided in the regulatory region required for full expression of the *Amy* distal gene identified by Hawley *et al.* (1992). Therefore it seems that expression of the duplicated *Amy* genes is coordinately controlled in part but the control elements are asymmetrically distributed in the intergenic region.

By using multiple alignment of 450 bp of the 5'-flanking region of the duplicated *Amy* genes from the eight sibling species in the *melanogaster* species subgroup, Okuyama *et al.* (1996) examined whether the putative regulatory elements identified from the nucleotide sequence of *D. melanogaster* (Boer and Hickey 1986; Hawley *et al.* 1992; Choi and Yamazaki 1994) were conserved among the species. They found that the several putative regulatory motifs, for example the TATA box, the CAAT box, the GATAAG sequence, which is a putative midgut-specific regulator (Magoulas *et al.* 1993a), and the CCAGTCAATAC/GGTCTGC sequence, which is thought to be involved in glucose repression (Boer and Hickey 1986), were conserved.

6. Adaptive AMY protein evolution in the *melanogaster* species subgroup

If molecular evolution occurs only by fixation of neutral mutations (Kimura 1983), the relative fixation probabilities of synonymous and replacement changes are expected to be the same between any stages of a phylogenetic tree. To test this null hypothesis, Shibata and Yamazaki (1995) performed a chi-square test of independence among four classes of nucleotide substitution. They classified all nucleotide substitutions in the coding region into either synonymous or replacement classes. They also classified all nucleotide substitutions into equal or not-equal classes, which were defined as new categories of the stages of a phylogenetic tree. When the proximal and distal genes of a species had the same substitution in all the eight species, the substitution was classified into the equal class; that is, the equal class must have the same substitution in both of the duplicated genes. The rest of the substitutions were classified into the not-equal class (figure 5). All substitutions therefore fell into four classes. Their test is similar to the G test in McDonald and Kreitman (1991), in which the categories of the stages of a phylogenetic tree are between-species and within-species classes.

Table 1 shows the 2×2 contingency table. A large excess of equal replacement substitutions compared with not-equal replacement substitutions was obtained from the close examination of the nucleotide substitutions, while synonymous substitution was observed in excess in the not-equal class. The chi-square value was highly significant and thus the null hypothesis was rejected. The test is more conservative when polymorphisms of *D. melanogaster* (Inomata *et al.* 1995a) are taken into consideration, since most of the within-species variation comprises not-equal substitution. However, the result remained unchanged. Therefore the simple neutral theory does not suffice to explain the pattern of nucleotide substitutions of the *Amy* genes among the eight sibling species of the *D. melanogaster* species subgroup.

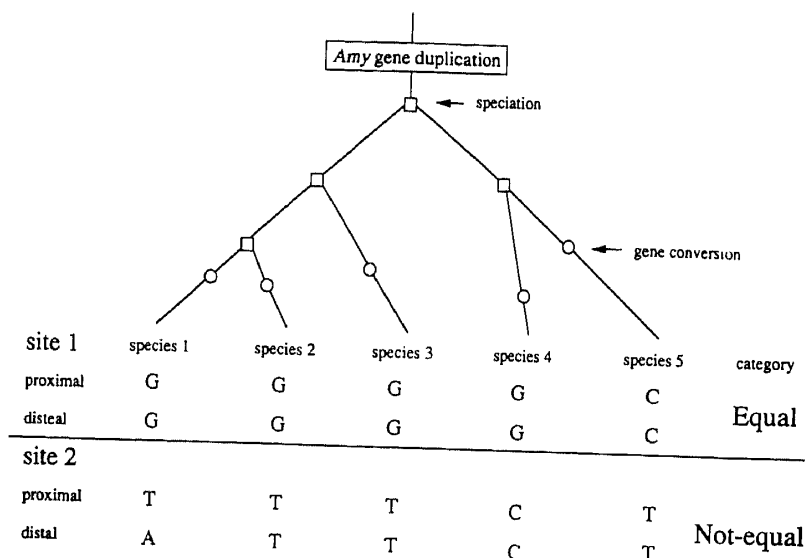


Figure 5. A model phylogenetic tree showing the timing of nucleotide substitutions and homogenization events leading to the equal or not-equal class of nucleotide substitution. The equal class may arise by substitutions occurring in any period before homogenizing events, where all species carry the same substitutions in both proximal and distal genes. The not-equal class is produced by substitutions occurring after homogenization events. At least one of the species has a different substitution in either the proximal or the distal gene. (From Shibata and Yamazaki 1995)

Table 1. Numbers of nucleotide substitutions in the four classes in the eight *D. melanogaster* subgroup sibling species^a.

	Replacement	Synonymous	Total
Equal	43	32	75
Not-equal	9	57	66
Total	52	89	141

^aFrom Shibata and Yamazaki (1995)

$\chi^2 = 28.8, P < 0.001$

The nucleotide substitutions specific to one species were also classified into four classes (table 2). These substitutions were considered to be ones that occurred in the lineage of each species after divergence from other species. The result of the test for independence performed for the eight species was also highly significant ($\chi^2 = 26.0$). Note that all species-specific substitutions occurred after speciation. In *D. erecta*, all seven equal substitutions were amino acid replacements. The ratio of replacement to synonymous substitutions in *D. erecta* ($100\% = 7/7$) was greater than the expected ratio (71%) under the assumption that all nucleotide substitutions were neutral, excluding termination codons (Nei 1975). Usually the ratio observed is much lower than the neutral expectation because of purifying selection. Indeed, the ratio was 21% in the case of the polymorphism data for the *Amy* genes in *D. melanogaster* (Inomata *et al.* 1995a). In *D. erecta* both *Amy* genes appear to be expressed and both AMY proteins encoded by them are putatively active, since their reading frames and the putative regulatory elements are well conserved. This indicates that amino acid substitution was highly accelerated in the lineage to *D. erecta*.

Table 2. Nucleotide substitutions specific to one species in the coding region of the *Amy* genes^a.

	Equal		Not-equal	
	Synon.	Repl.	Synon.	Repl.
<i>D. melanogaster</i> ^b	6	6	31	9
<i>D. simulans</i>	0	0	16	1
<i>D. mauritiana</i>	1	0	8	0
<i>D. sechellia</i> ^c	*	*	9	2
<i>D. erecta</i>	0	7	1	1
<i>D. orena</i>	4	11	2	1
<i>D. teissieri</i>	3	3	3	1
<i>D. yakuba</i>	3	2	1	0
Total	17	29	71	15

^aFrom Shibata and Yamazaki (1995)^bData from nine strains presented in Inomata *et al.* (1995a) except *Amy*^{null} strain^cOnly data for the proximal copy are shown.

Synon., synonymous; Repl., replacement

Shibata and Yamazaki (1995) proposed that the accelerated amino acid substitutions at the *Amy* locus are caused by fixation of adaptive mutations, as McDonald and Kreitman (1991) and Eanes *et al.* (1993) had done earlier. *D. melanogaster*, *D. erecta*, *D. teissieri* and *D. yakuba* exhibit different dependences on host plants under sympatric condition (Couturier *et al.* 1985). In particular, *D. erecta* feeds on only one genus of the Pandanaceae (screwpine, *Pandanus*), which is not used by other, sympatric species (Rio *et al.* 1983). Change of food sources in the process of speciation seems to play an important role in adaptive evolution of digestive enzymes such as α -amylase. It is quite natural to assume that adaptive replacement substitutions common to both of the duplicated genes have more effect on carriers than those in either of them. Then, the excess of replacement substitutions in the equal class can be easily understood in terms of adaptive AMY protein evolution.

To obtain supportive evidence of adaptation of the AMY protein, Shibata and Yamazaki (1994) examined several biochemical properties of α -amylase, such as specific activities under different temperature and pH ranges. They found several clear differences in the biochemical properties of the AMY proteins between species, although the differences were not sufficient to provide direct evidence of adaptation. Therefore detailed biochemical studies are necessary for obtaining direct evidence of adaptive AMY protein evolution.

7. Adaptive evolution of the regulatory region of the duplicated *Amy* genes in the *melanogaster* species subgroup

On the basis of the pattern of nucleotide divergence in the 5'-flanking region of the duplicated *Amy* genes, Okuyama *et al.* (1996) divided the 5'-flanking region into two regions, region 1 (immediately upstream of the *Amy* coding region) and region 2 (further upstream of the *Amy* coding region) (see figure 4). They found that the ratios

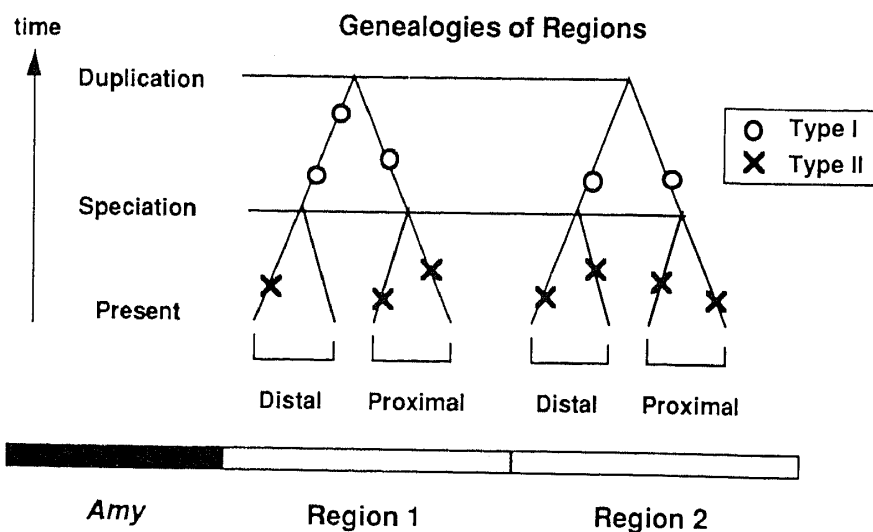


Figure 6. A model phylogenetic tree showing the timing of two classes of substitutions in region 1 and region 2. Substitutions that occurred between the duplication and speciation events (type I) are represented by circles and those that occurred after the speciation event (type II) are represented by crosses on the genealogies of respective regions. The region designated *Amy* is the coding region of the α -amylase gene.

Table 3. Numbers of type I and type II substitutions in region 1 and region 2 of the 5'-flanking region of the *Amy* genes in the *D. melanogaster* subgroup sibling species.^a

	Type I	Type II	Total
Region 1	62	119	181
Region 2	15	64	79
Total	77	183	260

^aFrom Okuyama *et al.* (1996)
 $\chi^2 = 6.16, P < 0.02$

of divergence between (proximal and distal) groups to those within the groups were different for region 1 and region 2. The divergence between the groups is due to substitutions that occurred after the gene duplication but before the initial diversification of the *D. melanogaster* species subgroup, while the divergence within the two groups is due to substitutions that occurred after diversification of the species subgroup. If the 5'-flanking region evolves through mutation and random genetic drift (Kimura and Ohta 1971; Kimura 1983) and the selective constraints are the same through time in region 1 and region 2, the two ratios should be equal.

To test the null hypothesis that the ratios of the two types of substitutions for regions 1 and 2 are equal, Okuyama *et al.* (1996) carried out a chi-square test of independence. Type I and type II substitutions were defined as those accumulated before (between groups) and after (within groups) the diversification of the *melanogaster* species subgroup respectively (figure 6). The numbers of the two types of substitutions were estimated from the phylogenetic tree constructed from data for the *Amy* coding regions by Shibata and Yamazaki (1995). The chi-square value was 6.16 (table 3). The null hypothesis that the ratios of type I substitutions to type II substitutions were the same

for regions 1 and 2 was rejected ($P < 0.02$). The result suggests that either there are non-neutral substitutions or the selective constraint has changed through time.

Since region 2 is divergent within groups (among species) and no putative regulatory elements are located in this region of the proximal genes, substitutions in this region are considered to be more likely to be neutral than those in region 1. In addition, some forces that promoted divergence between the proximal and distal sequences in region 1 seem to have worked. The following lines of evidence suggest differential functions of the two *Amy* genes. Region 1 was found to be more conserved than region 2 within a group (proximal or distal) and many putative regulatory elements were found in region 1. Magoulas *et al.* (1993a) indicated existence of multiple, functionally redundant elements for glucose repression in region 1 of the proximal gene in *D. melanogaster*. In addition, Magoulas *et al.* (1993b) showed that the 330-bp promoter region of the *D. virilis Amy* gene mediated glucose repression when it was transiently expressed in *D. melanogaster* larvae. These observations indicate the functional importance of region 1. Furthermore, independent regulation of components of the duplicated *Amy* locus was found in larvae of *D. melanogaster* (Klarenberg *et al.* 1986). Matsuo and Yamazaki (1986) showed that the duplicated *Amy* genes were in part differentially regulated by trans-acting factors. Therefore region 1 seems to have more potential for adaptive evolution. Accordingly, the most plausible model is that adaptive fixations of selectively advantageous mutations that differentiated expression of the proximal and distal genes occurred in region 1 after the duplication of the *Amy* gene, but before speciation.

Is it then true that substitutions in region 2 are selectively neutral? The evidence from the coding region suggests otherwise. Substitutions that occurred after species diversification were indeed selective (see table 1 and figure 5). Considering that substitutions in region 2 occurred in the same period as those in the coding region after speciation (see figure 6), it is probable that the substitutions in region 2 also are adaptive. More detailed studies along two lines—biochemical characterization of the AMY proteins and molecular characterization of the cis regulatory elements—should allow resolution of the hypothesis of adaptive evolution at the duplicated *Amy* locus in *Drosophila*.

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