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Evolution of the Response Patterns to Dietary Carbohydrates and the Developmental Differentiation of Gene Expression of α-Amylase in *Drosophila*

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Abstract. Intraspecific variation of α-amylase activity in *D. melanogaster* and *D. immigrans,* which is distantly related to *D. melanogaster,* and interspecific variation of α-amylase activity in 18 *Drosophila* species were examined. The amount of intraspecific variation of α-amylase activities measured in terms of coefficient of variation in *D. melanogaster* and *D. immigrans* was one-half and one-tenth or less, respectively, of the interspecific variation in 18 *Drosophila* species. We also surveyed the response patterns of α-amylase activity to dietary carbohydrates at the larval and adult stages. The levels of α-amylase activity depended on both repression by dietary glucose (glucose repression) and induction by dietary starch (starch induction). In general, our data suggest that glucose repression was conserved among species at both stages while starch induction was mainly observed in larvae, although the degree of the response depended on species. In *D. lebanonensis lebanonensis* and *D. serrata,* larvae expressed electrophoretically different α-amylase variants (isozymes) from those of adult flies. These results may suggest that the regulatory systems responsible both for the response to environment and developmental expression are different among species in *Drosophila.*

Key words: Evolution — Expression patterns — α-Amylase — Glucose repression — Starch induction — Intra- and interspecific variation — *Drosophila* — Gene expression — Regulatory genes

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Introduction

Evolution in regulatory genes has attracted the attention of many evolutionary biologists since changes in gene expression may provide raw materials for adaptive evolution (Dickinson 1991). For example, based primarily on the poor correlation between divergence of amino acid sequences and evolutionary changes at the phenotypic level, King and Wilson (1975) emphasized the importance of changes in regulatory genes rather than those in structural genes for adaptive evolution. However, only indirect approaches to this issue were possible in the past because regulatory genes could not be identified at the molecular level then. Recent advances in molecular biology provide good opportunities to study evolution of regulatory genes since now we can investigate effects of changes in regulatory sequences on gene expression directly. Careful interspecific surveys of interesting gene regulatory systems followed by molecular genetic analyses of the changes and evaluation of their adaptive significance will deepen our understanding of evolution in regulatory genes.

The α-amylase system in *Drosophila* provides a good model system for such studies. First, there is a large amount of genetic variation with respect to the levels of α-amylase in *Drosophila melanogaster* (Doane 1969;

Yamazaki and Matsuo 1984; Langley et al. 1988). Second, there is genetic variation in regulatory factors which 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, Yamazaki and Matsuo (1984) found a positive correlation between fitness and inducibility which is defined as representing the degree of amylase induction by dietary starch (starch induction). This indicates that the genetic variation for starch induction might be raw material for adaptive evolution. Third, the α-amylase (*Amy*) locus of all members of the *D. melanogaster* species subgroup is duplicated (Dainou et al. 1987; Payant et al. 1988; Shibata and Yamazaki 1995). Other species belonging to the subgenus *Sophophora* also have multiple copies of the amylase gene (Doane et al. 1987; Brown et al. 1990; Da Lage et al. 1992; Inomata, Tachida, and Yamazaki, unpublished result). Gene duplications followed by diversification of the copies with regard to gene expression and amino acid sequences are considered to be important factors for progressive changes of organisms (Ohno 1970; Ohta 1988). For these reasons, the *Drosophila Amy* gene system is considered to be suitable for the study of the evolution of regulatory genes.

Thus far, a few interesting evolutionary changes and conservations of the expression pattern in *Drosophila Amy* genes were reported. As for the changes, Powell et al. (1980) found that midgut patterns of amylase expression are highly variable both within and between species of the *obscura* and *willistoni* groups. Other changes include acquisition of dosage compensation associated with the translocation of the *Amy* gene to a novel X^2 chromosome in *D. miranda* (Norman and Doane 1990) and developmental changes of the expression pattern found in *D. ananassae* (Da Lage and Cariou 1993). However, Payant et al. (1988) showed that repression by glucose (glucose repression) of the *Amy* genes is a strongly conserved character through the *melanogaster* species subgroup. In fact, glucose repression is also found in *D. virilis,* which belongs to a different subgenus *Drosophila* from *D. melanogaster* (the subgenus *Sophophora*), and a transformation experiment showed that a similar system in the two species is responsible for the glucose repression (Magoulas et al. 1993). However, we still do not have a whole picture of the evolution of the *Amy* gene expression in *Drosophila.* These interesting features of the evolution described here certainly justify a systematic study of the *Amy* gene evolution in the genus *Drosophila.*

In the present study, we chose 28 representative species from the genus *Drosophila.* We surveyed expression patterns of the *Amy* genes in 20 out of these species on different media at larval and adult stages. Specific questions asked are: (1) how much difference is there among the species; (2) to what extent is glucose repression and starch induction found in *D. melanogaster* conserved; and (3) are there any changes in stage specificity of gene expression? In order to complement the interspecific comparison, we also surveyed intraspecific variation of the *Amy* gene activities using 18 lines of *D. melanogaster* representing the subgenus *Sophophora* and 13 lines of *D. immigrans* representing the subgenus *Drosophila.* We report the results of these surveys in this article.

Materials and Methods

Drosophila Species. The *Drosophila* species used in this study are listed in Table 1. A single isofemale line was used for each species except for *D. melanogaster* and *D. immigrans. D. ficusphila, D. lebanonensis lebanonensis, D. lini, D. saltans, D. serrata, D. miranda,* and *D. persimilis* were obtained from the National *Drosophila* Species Resource Center at Bowling Green State University. *Chymomyza procnemis, Zaprionus indianus, D. albomicans, D. americana americana, D. annulipes, D. auraria, D. barbarae, D. bifasciata, D. bocki, D. busckii, D. elegans, D. eugracilis, D. funebris, D. hydei, D. immigrans, D. kikkawai, D. pseudoobscura, D. sordidula, D. takahashii,* and *D. virilis* were obtained from Tokyo Metropolitan University. Species are chosen so that at least one species from each main species group of *Drosophila* is represented in the sample.

Eighteen isofemale lines of *D. melanogaster* were used in this study. They are: A2, A3, A11, and A15 (Asakura, Japan, 1990); ta11 and ta12 (Tanushimaru, Japan, 1990); fu11, fu27, to56 and to60 (Mishima, Japan, 1990); I293, I297, and I319 (Ishigaki Island, Japan, 1990); Og54, Og115, and Og257 (Ogasawara Is., Japan); j70 (Tananarive, Madagascar); and KO11 (Gwacheon, Korea). Twelve of 13 isofemale *D. immigrans* lines used in this study were collected in Fukuoka, Japan, in 1993 and one strain (551-5) was obtained from Tokyo Metropolitan University.

Compositions of Three Different Media. Ebios medium (control medium): 5% ebios (killed yeast) (w/v), 0.6% agar (w/v), and 0.4% propionic acid (v/v) in distilled water. Glucose medium (treatment medium): 10% glucose (w/v), 5% ebios (w/v), 0.6% agar (w/v), and 0.4% propionic acid (v/v) in distilled water. Starch medium (treatment medium): 10% soluble starch (w/v), 5% ebios (w/v), 0.6% agar (w/v), and 0.4% propionic acid (v/v) in distilled water. The control medium and the treatment media have identical composition except for the addition of a specific carbohydrate (glucose or starch) in each treatment medium. Cornmeal medium is used to maintain stocks in our laboratory. Larvae and adult flies used for electrophoresis were also raised on cornmeal medium. Cornmeal medium: 7% cornmeal (w/v), 10% glucose (w/v), 5% ebios (w/v), 0.6% agar (w/v), and 0.4% propionic acid (v/v) in distilled water.

Sample Collection. Adult flies were transferred to vials containing the control or either one of the treatment media and allowed to lay eggs at 22°C. Five third-instar larvae were randomly collected from a vial without distinguishing sexes, washed with distilled water, and then frozen at −70°C. Adult flies eclosed from a vial from which larvae were collected were transferred to a new vial with the same medium and allowed to feed for 3–5 days. Three to five adult flies were randomly collected from each vial without distinguishing sexes and frozen at −70°C. Those constitute one replicate of the sample. Three replicates of larvae and adult flies were prepared per line (species) on each medium.

Activity of α-Amylase and Protein Assay. Larvae or adult flies were homogenized with 500 μl of distilled water by sonication. The ho-

Table 1. *Drosophila* species used in this study

Genus	Subgenus	Species group	Species subgroup	Species
Chymomyza				*Ch. procnemis*
Zaprionus	Zaprionus			*Z. indianus*[a]
Drosophila	Scaptodrosophila	victoria		*D. lebanonensis lebanonensis*
	Dorsilopha			*D. busckii*
	Sophophora	melanogaster	melanogaster	*D. melanogaster*
			takahashii	*D. takahashii*
			ficusphila	*D. ficusphila*[a]
			montium	*D. auraria*
				D. kikkawai
				D. barbarae
				D. bocki
				D. lini
				D. serrata[a]
			elegans	*D. elegans*[a]
			eugracilis	*D. eugracilis*
		obscura		*D. bifasciata*[a]
			pseudoobscura	*D. pseudoobscura*
				D. miranda
				D. persimilis
		saltans	saltans	*D. saltans*
	Drosophila	virilis	virilis	*D. virilis*
				D. americana americana
		robusta		*D. sordidula*[a]
		repleta		*D. hydei*
		funebris		*D. funebris*
		immigrans	immigrans	*D. immigrans*
			nasuta	*D. albomicans*[a]
			hypocausta	*D. annulipes*[a]

[a] Species used on' · electrophoresis

mogenates were centrifuged at 10,000 rpm for 5 min, and the supernatants were assayed for enzyme activity and protein content.

The commercially available kit, amylase B-testwako (Wako), which applies the carboxymethyl (CM)-amylose DEX method, was used to measure α-amylase activity. We can measure α-amylase activity alone by the kit; 250 μl of the enzyme solution in the kit (26 U/ml exo-1,4-α-D-glucosidase, 56 U/ml glucose oxidase, 0.3 U/ml mutarotase, 450 U/ml catalase, 0.3 U/ml ascorbic acid oxidase, 10 mM MES buffer [pH 6.0] and *N,N*-diethyl-3,5-xylidine [DEX]) were added to 20 μl of the supernatant and incubated for 5 min at 37°C. Then 250 μl of the substrate solution in the kit (5.8 mg/ml CM-amylose, 0.95 mmol/l 4-aminoantipyrine, 5.7 U/ml peroxidase and 0.1 M MES buffer [pH 7.0]) was added. After incubation for 10 min at 37°C, 500 μl of the stop solution (0.6 M Tris buffer [pH 7.4] and EDTA) was added to the reaction. Activity of α-amylase was quantified spectrophotometrically by measuring the absorbance at 620 nm.

The commercially available kit BCA protein assay reagent (Pierce) was used to measure the protein content; 2 ml of the BCA working reagent was added to 50 μl of the supernatant. After incubation for 30 min at 37°C, protein content was measured spectrophotometrically with absorbance at 562 nm. Activity of α-amylase is expressed by glucose units, where 1 glucose unit represents 10^{-4} μmol glucose released per minute at 37°C. Specific activity of α-amylase is defined as α-amylase per μg protein.

Electrophoresis. Several third-instar larvae and adult flies of each species were homogenized by sonication in a buffer (pH 8.9) (0.1 M Tris-borate, 5 mM $MgCl_2$ and 10% sucrose [w/v]). Those samples were immediately applied to the polyacrylamide gels (5% acrylamide [w/v], 0.2% bis-acrylamide [w/v], 20 mM $CaCl_2$, and 0.1 M Tris-borate) in a 0.1 M Tris-borate (pH 8.9) buffer. After running for 3 h at 0°C, 300 V, the gels were incubated in starch solution (2% soluble starch [w/v], 0.1 M Tris-HCl [pH 7.4], and 20 mM $CaCl_2$) for 2 h at 37°C. They were washed with water briefly and incubated again in a solution without starch (0.1 M Tris-HCl [pH 7.4] and 20 mM $CaCl_2$) for 1 h at 37°C (Benkel and Hickey 1986a). They were then washed with water and stained in I_2-KI solution. The band mobilities were measured with reference to those of standard marker strains of *D. melanogaster,* TN329 (AMY^1) and 1420#1 ($AMY^{4,6}$) (Inomata et al. 1995). There were the following minor differences in electrophoretic conditions for *D. ficusphila* and *D. serrata* (Fig. 4B) from that for the other *Drosophila* species; a single larva or adult fly was homogenized and applied to the gel so that we could observe interindividual variations in activity. After running (about 4–5 h) at 5°C, 170 V, the gels were incubated for 30–45 min in 1% soluble starch solution (1% soluble starch [w/v], 0.1 M Tris-HCl [pH 7.4] and 20 mM $CaCl_2$). Gels were then washed and stained in I_2-KI solution.

Data Analyses. Statistical analyses were carried out using the SYSTAT software version 5.2 (Macintosh). Activity data were analyzed using the two-way analysis of variance. In the analyses of variance of intraspecific α-amylase activities, line effects were considered as random effects. Species effects in the analysis of interspecific α-amylase activities and medium effects in all of the analyses of variance were considered as fixed effects. As described here, since samples of adult flies were collected from the same vial as that from which larvae were collected, adult and larval measurements in each replicate are not independent each other. Therefore, in order to examine correlations between larvae and adults we used only a part of the data, so these two values become independent. The results were similar to those calculated by using averages of three replicates. Correlations between the activity of flies raised on glucose environments and that on starch environments were computed using the average values.

Results

Variation of α*-Amylase Activity on Different Media in* Drosophila *Species*

We surveyed α-amylase-specific activities on three different media in 18 *Drosophila* species in addition to *D. melanogaster* and *D. immigrans* described here (Fig. 1). Average specific activities of 18 *Drosophila* species in glucose, ebios (control), and starch media were 0.223, 0.576, and 0.851 (glucose units/μg protein) at the larval stage and 0.718, 0.918, and 0.882 (glucose units/μg protein) at the adult stage, respectively. Specific activities of α-amylase were significantly different among 18 species (Table 2). These results suggest that specific activities of 18 *Drosophila* species are distributed in a wide range. The medium effects were also significant (Table 2). Since glucose and starch media were made by adding the carbohydrates, glucose and starch, respectively, to the ebios medium, the specific effects of the dietary carbohydrates, i.e., glucose repression and starch induction, could be assessed separately. Tukey's HSD simultaneous pairwise mean comparison is performed to examine which means differ from others. In other words, we used this test to statistically identify the effect by a specific dietary carbohydrate. The effects of glucose were significant at the larval and adult stages ($P \ll 0.01$). The differences between the glucose and ebios environments at the larval and adult stages were –0.353 and –0.200 (glucose units/μg protein), respectively. The effect of starch was significant at the larval stage ($P \ll 0.01$) but not significant at the adult stage ($P = 0.729$). The differences between the starch and ebios environments at the larval and adult stages were 0.275 and –0.036 (glucose units/μg protein), respectively. These results suggest a general picture with respect to the response pattern to the dietary carbohydrates in *Drosophila*—namely, glucose generally reduces the activities at both larval and adult stages while starch increases the activities mainly at the larval stage in *Drosophila* species. However, since the interaction effects were also highly significant at both stages, the magnitudes of responses to carbohydrates differed among species. For example, *D. kikkawai* appeared to be the most inducible species by starch in both larvae and adults at face value.

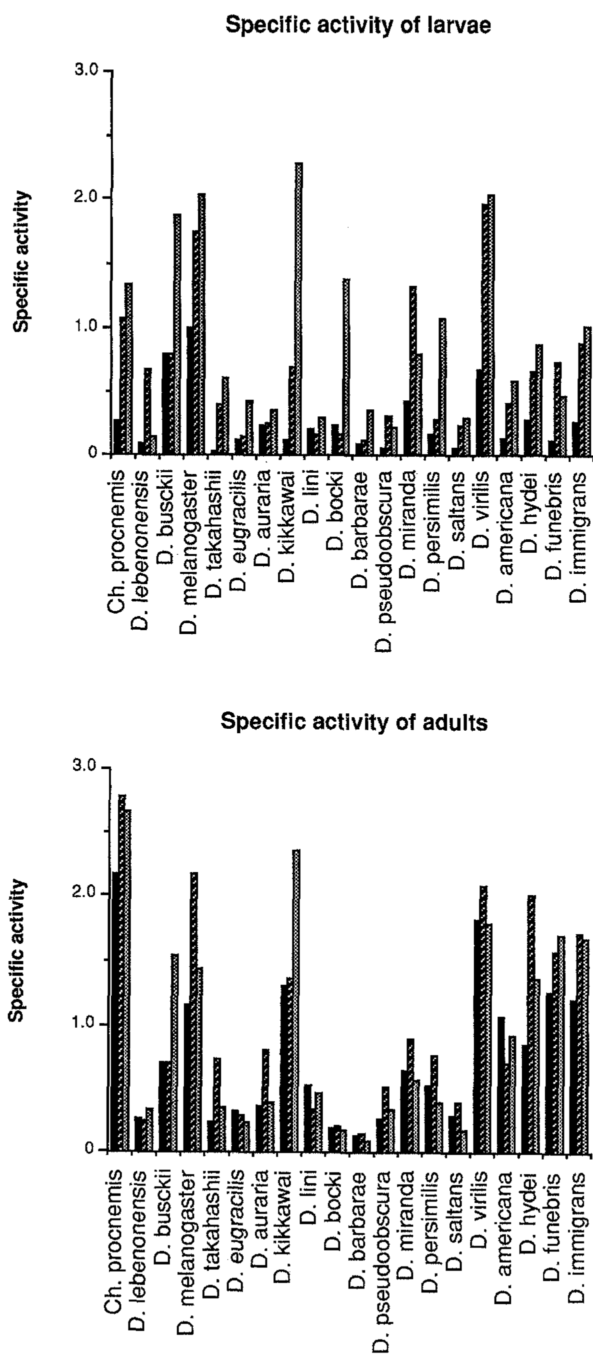

Fig. 1. Specific activity of α-amylase (glucose units/μg protein) of 20 *Drosophila* species in larvae (top) and adults (bottom) on glucose (■), ebios (control) (▨), and starch medium (▤). Specific activities of *D. melanogaster* and *D. immigrans* are average of intraspecific activities.

Variation of α*-Amylase Activity on Different Media in* D. melanogaster

Eighteen isofemale lines of *D. melanogaster* from natural populations were raised on the three different media. Specific activities of α-amylase of the larvae and adult flies were examined. On the average, α-amylase-specific activities in glucose, ebios, and starch media of larvae were 1.002, 1.746, and 2.028 (glucose units/μg protein), respectively. Specific activities of α-amylase in glucose, ebios, and starch media of adult flies were 1.153, 2.159 and 1.430 (glucose units/μg protein), respectively. There were significant line effects (Table 3). This means that there is genetic variation with respect to α-amylase-specific activity in *D. melanogaster*. This result is consistent with those of the previous reports (Yamazaki and Matsuo 1984; Matsuo and Yamazaki 1984).

Significant medium effects were also found (Table 3). The difference in average specific activities between glucose and ebios media were –0.745 ($P \ll 0.01$) (glucose units/μg protein) at the larval stage and –1.006 ($P \ll$

Table 2. Two-way analysis of variance of α-amylase specific activity in 18 *Drosophila* species

Source	*df*	Mean square	*F*
Third-instar larvae			
Species	17	1.3917	11.3**
Medium	2	5.3491	43.6**
Interaction	34	0.3836	3.1**
Error	106	0.1228	
Adult flies			
Species	17	4.1384	73.9**
Medium	2	0.5965	10.7**
Interaction	34	0.2042	3.6**
Error	104	0.0560	

** Highly significant ($P < 0.01$)

Table 4. Two-way analysis of variance of α-amylase specific activity in *D. immigrans*

Source	*df*	Mean square	*F*
Third-instar larvae			
Line	12	0.3942	2.9**
Medium	2	6.0469	23.5**
Interaction	24	0.2577	1.9*
Error	76	0.1363	
Adult flies			
Line	12	0.8398	4.8**
Medium	2	2.9768	18.1**
Interaction	24	0.1644	0.9
Error	72	0.1766	

* Significant ($P < 0.05$)
** Highly significant ($P < 0.01$)

0.01) at the adult stage. The difference in average specific activities between starch and ebios media at the larval and adult stages were 0.282 ($P < 0.01$) and –0.729 ($P \ll 0.01$) (glucose units/μg protein), respectively. These results show that at the larval stage α-amylase expression is repressed by dietary glucose (glucose repression) and is induced by dietary starch (starch induction). In contrast, the level of α-amylase activity was significantly lowered by both dietary glucose and starch at the adult stage, although the level of α-amylase activity on glucose medium was significantly lower than that on starch medium when the pairwise mean comparison was performed (difference was 0.277, $P \ll 0.01$). Thus, the response pattern at the adult stage to starch in *D. melanogaster* was different from that of the general picture in *Drosophila* species mentioned here.

Variation of α-Amylase Activity on Different Media in D. immigrans

Specific activities of α-amylase of 13 isofemale lines of *D. immigrans* from natural populations were also examined on three different media. The average specific activities in glucose, ebios, and starch media were 0.245, 0.848, and 0.999 (glucose units/μg protein) at the larval stage and 1.225, 1.750, and 1.678 (glucose units/μg protein) at the adult stage, respectively. The lines effects were significant (Table 4). The medium effects were also significant (Table 4). The differences in average specific activities between glucose and ebios media were –0.603 ($P \ll 0.01$) at the larval stage and –0.525 ($P \ll 0.01$) at the adult stage. The differences between starch and ebios media were 0.151 ($P = 0.180$) at the larval stage and –0.072 ($P = 0.753$) (glucose units/μg protein) at the adult stage. These results may suggest that in *D. immigrans* there is no starch induction at any developmental stages, although there is glucose repression at both developmental stages. Accordingly, the response pattern to the dietary carbohydrates of the larval stage is similar to that of the adult stage in *D. immigrans.*

Table 3. Two-way analysis of variance of α-amylase specific activity in *D. melanogaster*

Source	*df*	Mean square	*F*
Third-instar larvae			
Line	17	5.8243	28.9**
Medium	2	14.4091	24.9**
Interaction	34	0.5795	2.9**
Error	103	0.2018	
Adult flies			
Line	17	5.8560	56.5**
Medium	2	13.8145	64.4**
Interaction	34	0.2145	2.1**
Error	102	0.1036	

** Highly significant ($P < 0.01$)

Intra- and Interspecific Variation for α-Amylase Activity

We compared the amounts of intra- and interspecific genetic variations for α-amylase activity in terms of the coefficient of variation [variance/(square of mean)]. The genetic variances were computed by [Mean Square (line or species) – Mean Square (error)]/3 from the analysis of variance table. The coefficients of variation in larvae are 1.40 (among species), 0.74 (*D. melanogaster*), and 0.18 (*D. immigrans*). Those in adults are 1.87 (among species), 0.75 (*D. melanogaster*), and 0.09 (*D. immigrans*). Thus, *D. melanogaster* and *D. immigrans* have about one-half and one-tenth or less, respectively, of the genetic variation found among species if we measure variation in terms of the coefficient of variation.

Correlation Between Larvae and Adults

In order to see whether the same factors are responsible for the amylase activity variation in larvae and adults, we computed the correlation of specific activities in larvae and adults. In this analysis, we removed *D. melanogaster* lines with $AMY^{3,6}$ whose catalytic efficiency is known to be high (Doane 1969) in order to eliminate the correlation due to this high-activity isozyme. In *D. melano-*

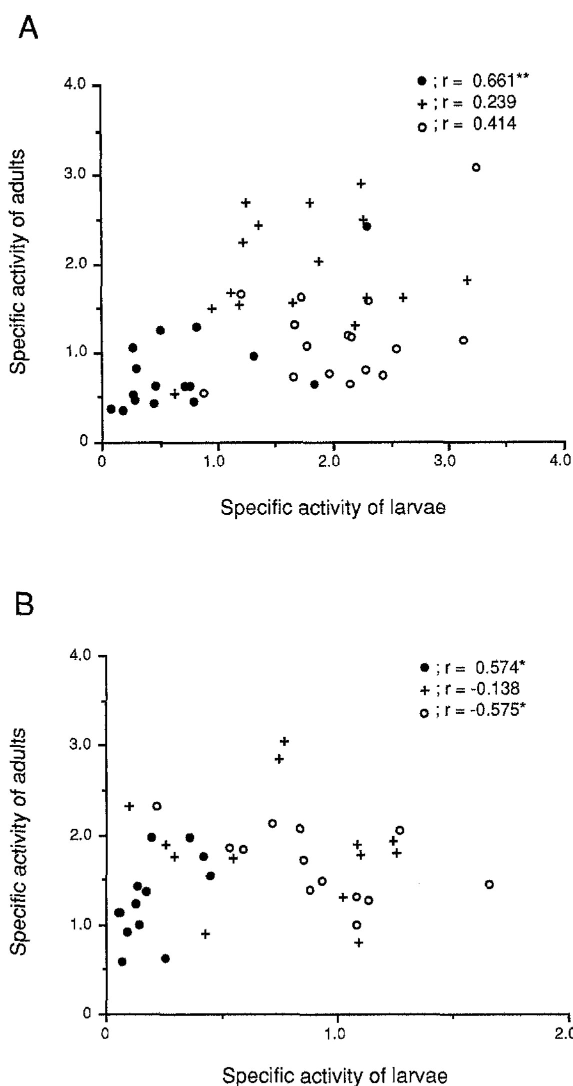

Fig. 2. Correlation between larval and adult specific activities (glucose units/μg protein) on glucose (●), ebios (control) (+), and starch (○) medium in *D. melanogaster* (**A**) and *D. immigrans* (**B**). Significant at 5% level (*) and significant at 1% level (**).

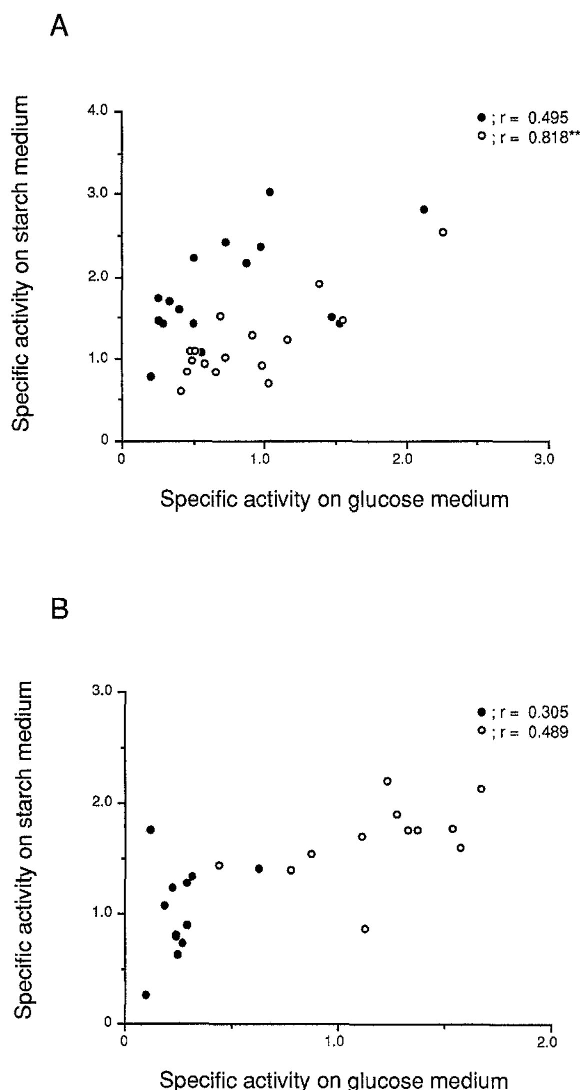

Fig. 3. Correlation between specific activities (glucose units/μg protein) on glucose and starch media in *D. melanogaster* (**A**) and *D. immigrans* (**B**). Specific activities in larvae (●) and adults (○). Significant at 1% level (**).

gaster the α-amylase specific activity of larvae positively correlated with that of adults on glucose medium ($r = 0.661$, $P < 0.01$). On the two other media, correlations were not significant ($r = 0.239$, $P > 0.05$ on ebios medium, $r = 0.414$, $P > 0.05$ on starch medium; see Fig. 2). In *D. immigrans* correlation between α-amylase specific activities of larvae and adults were $r = 0.574$ ($0.01 < P < 0.05$) on glucose medium, $r = -0.138$ ($P > 0.05$) on ebios medium, and $r = -0.575$ ($0.01 < P < 0.05$) on starch medium (Fig. 2).

Correlation of Activities in Glucose and Starch Environments

In order to examine whether the same factors are responsible for glucose repression and starch induction, we computed the correlation between activities in starch and glucose environments. By the same reasoning as that in the previous section, we removed *D. melanogaster* lines with $AMY^{3,6}$ from the analysis. The results are shown in Fig. 3. In *D. melanogaster,* the correlations are $r = 0.495$ ($P \approx 0.05$) in larvae and $r = 0.818$ ($P < 0.01$) in adults. In *D. immigrans,* the correlations are $r = 0.305$ ($P > 0.05$) in larvae and $r = 0.489$ ($P > 0.05$) in adults. Although all correlations are positive, only those in *D. melanogaster* were significant.

Electrophoresis

We surveyed 21 *Drosophila* species by electrophoresis to visually detect differences of expressed isozymes at the larval and adult stages. Figure 4 shows the result. We found that *D. lebanonensis lebanonensis* and *D. serrata* exhibited bands with different mobilities (isozymes) at the larval and adult stages. For *D. ficusphila* and *D. serrata,* we prepared homogenates from a single larva or

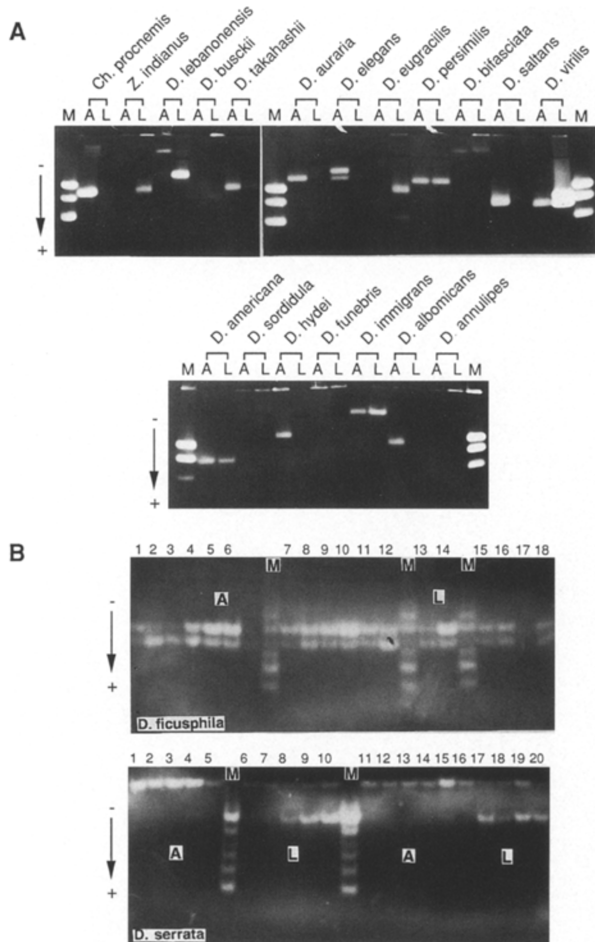

Fig. 4. The stage-specific expression patterns in *D. Drosophila.* L: larvae, A: adult flies. **A** Several individuals were used per assay. The pattern of expressed isozymes of larva is different from that of adult in *D. lebanonensis lebanonensis.* The AMY^1, AMY^4, and AMY^6 isozymes of *D. melanogaster* were used as mobility markers (M). **B** A single individual was used per assay. The pattern of expressed isozymes of larva is the same as that of adult in *D. ficusphila* but not in *D. serrata.* The AMY^1 through AMY^6 isozymes of *D. melanogaster* were used as mobility markers (M). In *D. ficusphila,* lanes 1–6 and 15–18: adult and lanes 7–14: larva. In *D. serrata,* lanes 1–5 and 11–16: adult and lanes 6–10 and 17–20: larva.

adult fly in order to observe interindividual variations (Fig. 4B). There were interindividual variations of relative band intensity in *D. ficusphila.* For example, the intensity of the slower band is much stronger than that of the faster band in lane 10 but the two bands have almost the same intensity in lane 12. Note that band intensities do not necessarily correlate with the specific activities in Fig. 1 because band intensities depend on the amount of homogenates assayed. Therefore, the specific activities and absolute band intensities shown in Fig. 4 are not directly comparable.

Discussion

Many species other than *D. melanogaster* and *D. immigrans* showed low activities in Fig. 1. Since we used a commercially available kit, the assay conditions such as pH and temperature might not have been optimal for isozymes in some species, and this might be the reason for the low activities observed. Here we briefly consider the effect of low activity on the evaluation of the magnitude of variation. Roughly speaking, enzyme activity is considered to be affected multiplicatively by the catalytic efficiency. Therefore, if the catalytic efficiency of the enzyme is low due to the experimental condition used and we measure variation by variance, the genetic variation may be underestimated. This underestimation can be rectified by using coefficient of variation, which is defined as the variance divided by the square of the mean because the effect of low catalytic efficiency is cancelled out by appearing both in denominator and numerator. In the present study, we used coefficient of variation to measure intra- and interspecific variation of specific activities for this reason. Although the rectification is only partial because the catalytic efficiency in a specific environment may differ among species, relative variations around the mean were quantified and we could compare the intra- and interspecific in a more meaningful way.

We did not distinguish sexes of individuals in the samples in the present study. There might be sexual differences of amylase activities though our preliminary study indicates that differences are not much. Even if there are differences, we independently sampled individuals for three replicates, and sexual differences should be included in the differences among replicates. Our conclusion from the analysis of variance is based on the main effects and interaction which are not influenced by the difference among replicates and thus is not affected by the sexual differences.

Although there are some exceptions, it is worthwhile to make some generalizations concerning the evolution of the amylase system in *Drosophila* from our analysis-of-variance data. First, glucose repression is a well-conserved trait through the developmental stages, while starch induction is observed mainly at the larval stage. Amylase expression of *D. miranda, D. persimilis,* and *D. virilis* is previously reported to be repressed by dietary glucose at the larval and adult stages (Norman and Doane 1990; Magoulas et al. 1993). Second, the degree of induction or repression differs among species since a significant species × medium interaction was found. This can be also seen from Fig. 1. For example, in *D. busckii* and *D. lini,* glucose repression may be very weak, if there is any at all. In fact, at face value, the activity was higher in the glucose environment than that in the ebios environment in the latter species. However, the difference was not significant, and we think the glucose induction was not real. Another example is the starch repression in adults of *D. melanogaster.* Therefore, *Drosophila* amylase systems seem to be evolving, maintaining glucose repression and larval starch induction but modifying their magnitudes to adapt to environments in which respective species live.

In order to conduct a systematic survey, specific activities of 20 species from several major-species groups of *Drosophila* were examined. Although the observed specific activity of each species is not necessarily the species average since only one isofemale line per species was examined, it may be worthwhile to examine these evolutionary changes in *Amy* gene expression, taking into account the information on the taxonomic relationship of the species. The closest relationships of species in our study were those among the species subgroups. In this study the within-species subgroup comparison was made in the *montium,* the *pseudoobscura,* and the *virilis* species subgroups. Even among sibling or closely related species, there are considerable changes in the *Amy* expression pattern. For example, in the *montium* species subgroup, *D. kikkawai* showed relatively high specific activity and striking response to dietary carbohydrates such as glucose repression and starch induction at face value. In contrast, specific activities and magnitudes of response of some members of the *montium* species subgroup (*D. auraria, D. barbarae,* and *D. lini*) were at the lowest level in both larval and adult stages in the *Drosophila* species examined. In the *pseudoobscura* species subgroup, the response pattern to dietary carbohydrates of *D. miranda* was very similar to that of *D. pseudoobscura.* The two species did not have starch induction at face value through the developmental stages while there was starch induction at the larval stage in their close relative, *D. persimilis.* The sequence divergence of the *Amy* gene between *D. persimilis* and *D. miranda* or *D. pseudoobscura* is about 1–2% per synonymous site (Inomata, Tachida, and Yamazaki, unpublished result). This value is smaller than or comparable to that of the sequence divergence within *D. melanogaster* (about 3.7%, Inomata et al. 1995). The low sequence divergence indicates recent diversification of these three species, and the change of the expression pattern is considered to have occurred fairly recently. The two species in the *virilis* species subgroup also differ in their expression pattern. Thus, changes in gene regulation seem to have taken place frequently even among closely related species. However, the same expression patterns were observed among distantly related species. For example, *D. busckii,* which is in the subgenus *Dorsilopha,* and *D. kikkawai* in the subgenus *Sophophora* showed starch induction at the adult stage, while adults of most *Drosophila* species have no starch induction, as mentioned earlier. In order to answer questions such as whether the starch induction is an ancestral trait based on the same genetic system or not and whether changes are adaptive or not, further studies to characterize the regulatory systems of amylase and the ecological niches of the species are necessary.

In the glucose environment, amylase activities of larvae and adults were positively correlated both in *D. melanogaster* and *D. immigrans.* In this environment, some common factors seem to control amylase activities although we do not know whether the factors are structural or regulatory. There was a negative correlation between the levels of α-amylase activity in different developmental stages on ebios and starch media in *D. immigrans.* The correlation on starch medium was statistically significant. If differences in catalytic efficiencies of isozymes are responsible for the variations at respective developmental stages, we should observe positive correlations. Thus, *D. immigrans* may have some regulatory elements which act in the opposite directions in the two developmental stages.

As for activities in glucose and starch environments, we found positive correlations in adults and larvae of *D. melanogaster* and *D. immigrans.* In the former species, the correlations were statistically significant. Common factors which act in both environments seem to control amylase activities. Again factors may be regulatory or structural and further molecular analyses are necessary to characterize them.

We obtained direct evidence of differentiation of α-amylase expression between the two stages in *D. lebanonensis lebanonensis* and *D. serrata* (Fig. 4). The regulatory systems of α-amylase expression of *D. lebanonensis lebanonensis* and *D. serrata* seem to have different components from that of other species examined. Expression patterns of these two species may suggest that the *Amy* loci of *D. lebanonensis lebanonensis* and *D. serrata* consist of at least two copies and that stage-specific genes are expressed. Alternatively, a stage-specific isozyme may be expressed from a single gene by alternative splicing. Stage-specific differentiation of *Amy* gene expression is also reported in *D. ananassae* (Da Lage and Cariou 1993). In *D. ananassae,* the *Amy* locus consists of two independent blocks on different chromosomal positions, each block containing duplicated genes (Da Lage et al. 1992). Different blocks are thought to be used in a stage-specific manner (Da Lage and Cariou 1993). Changes in gene organization followed by functional diversification of structural genes (Hood et al. 1975; Ohta 1988) and differentiation in gene regulation (King and Wilson 1975) are considered to be important to acquire complexity of higher organisms. In this respect, the α-amylase system in *Drosophila* is one of the simplest models available that can be used to obtain a better understanding of adaptive evolution, and our present survey extended the knowledge on evolution of the α-amylase system in *Drosophila,* revealing various modes of changes.

In conclusion, we have shown that the levels of α-amylase activity of various species had a wide range and were subject to both glucose repression and larval starch induction in the majority of the species. We also found that the degrees of the response to environments were differentiated among species and the expression patterns of α-amylase differed not only between species but also between stages. However, the molecular basis and the selective significance of these changes in regu-

latory patterns are not known and we need to clarify them in future studies.

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