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Effect of nucleotide polymorphism in *cis*-regulatory and coding regions on amylase activity and fitness in *Drosophila melanogaster*

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In natural populations of *Drosophila melanogaster*, there are many amylase (AMY) isozymes encoded by the duplicated genes, but their adaptive significance remains unclear. One approach to elucidate this issue is to understand the molecular basis of functional differences between the allelic classes. In this study, the effects of nucleotide polymorphism in 5′-flanking (*cis*-regulatory) and coding regions on AMY activity were examined, both on glucose and starch food media and in larvae and adults, using three chimeric *Amylase* (*Amy*) genes, Amy^{c111}, Amy^{c161} and Amy^{fc661}. In this notation, the first number in the superscript indicates the sequence of the 5′-flnaking region (similar to Amy^{1} or Amy^{6}), the second number refers to the coding region and the third number to the 3′-flanking region. We found that effect of nucleotide polymorphism in the coding region differed between larvae and adults. In larvae, the coding sequence of the Amy^{6} allele resulted in higher AMY activity than that of Amy^{1} allele, indicating the post-transcriptional differences between them. The *cis*-regulatory region derived from the Amy^{6} allele resulted in higher AMY activity in both larvae and adults. Thus, two fitness components, developmental time and productivity, were measured to examine whether polymorphism in the *cis*-regulatory region between the two alleles has an effect on them, but no significant difference was detected. We raise the implications for the evolution of subfunctionalization in multigene families.
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Keywords: *Drosophila*; amylase; multigene family; evolution; polymorphism; *cis*-regulatory region

Introduction

Since the findings of the unexpectedly high amount of protein polymorphism (allozymes) in various organisms in nature (eg Harris, 1966; Hubby and Lewontin, 1966; Lewontin and Hubby, 1966), the mechanisms explaining its maintenance have been extensively discussed. At the protein level, most observations appear to be consistent with the neutral hypothesis. As it has become easy to determine nucleotide sequences, the main interest has shifted to understanding maintenance mechanisms of DNA variation rather than those of protein polymorphism. As a result, the adaptive significance of allozyme polymorphism remains unclear.

One approach to evaluate adaptive significance of allozyme polymorphism is to understand the molecular basis of functional differences between the allelic classes. In this context, the most advanced study is Fast and Slow allozyme polymorphism of alcohol dehydrogenase (*Adh*) gene in *Drosophila melanogaster*. Association studies and experimental approaches *in vivo* clearly showed differences in catalytic efficiency as well as in noncoding regions between the two allelic classes (eg Aquadro *et al*, 1986; Laurie and Stam, 1988, 1994; Choudhary and Laurie, 1991; Laurie *et al*, 1991; Berry and Kreitman, 1993; Stam and Laurie, 1996).

In contrast to extensive progress of the studies on allozyme polymorphism from a single locus such as the *Adh* gene in *Drosophila*, examinations of the adaptive significance of isozymes, which are proteins produced by different loci, have been lacking. Since the level of activity and protein content of an individual (strain) is the sum over all the isozymes, most experimental approaches that have been used in single-gene analysis cannot be applied directly to the products of duplicated genes. Instead, we have made use of *P*-element-mediated transformation to enable us to dissect effects of individual isozymes produced by such genes.

The starch degradation of *Drosophila* is one of the most extensively studied processes in the evolutionary research. The α-amylase (AMY, EC 3.2.1.1) breaks down starch into glucose and maltose. *D. melanogaster* has two *amylase* (*Amy*) gene copies, composed of the *Amy-proximal* and *Amy-distal* genes (Gemmill *et al*, 1985; Levy *et al*, 1985). The AMY activity is repressed by its products, glucose and maltose (Doane, 1969; Hickey and Benkel, 1982; Inomata *et al*, 1995a) and induced by the substrate, starch (Inomata *et al*, 1995a). Variation in AMY activity and food-response ability (inducibility) has been found (Matsuo and Yamazaki, 1984, Yamazaki and Matsuo, 1984, Inomata *et al*, 1995a). Moreover, food-response ability positively correlates with fitness components

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(Matsuo and Yamazaki, 1984; Yamazaki and Matsuo, 1984), indicating that regulatory factors controlling *Amy* gene expression are important for adaptation.

In natural populations of *D. melanogaster*, there are six major and many minor AMY isozymes (Kikkawa, 1964; Doane, 1969; Dainou *et al*, 1987; Inomata *et al*, 1995b). There are considerable differences in the level of AMY activity among strains with different isozymes (Kikkawa, 1964; Doane, 1969). Hoorn and Scharloo (1978) showed differences in the K_m (enzyme–substrate affinity, the Michaelis constant) and V_{max} (maximal activity) between AMY[1] and AMY[4,6] strains, and suggested some of the activity differences were due to the catalytic efficiency of the enzymes. On the other hand, Hickey (1981) showed a difference in the amount of amylase protein between high- and low-activity AMY[1] variants. Yamate and Yamazaki (1999) found a positive correlation between specific activity and the amount of mRNA among strains with different AMY isozymes. On the basis of these observations, the variation of specific activity between AMY isozymes is likely to be largely explained by the differences in the amount of enzymes. The previous studies did not control the genetic backgrounds of the strains with different AMY isozymes and did not examine each isozyme and gene copy separately. Therefore, it is possible that they could not properly evaluate effects of individual isozymes. Moreover, molecular dissection of the effects of activity variation between the AMY isozymes has not been done yet.

To clarify the causes of the AMY activity differences among fly strains with different AMY isozymes, we examined the effects of differences in the nucleotide sequence between two alleles, *Amy[1]-distal* and *Amy[6]-distal*, of one of the duplicated *Amy* genes. We show that nucleotide polymorphism in both *cis*-regulatory and coding regions affects AMY activity. We also assessed the effect of nucleotide polymorphism in *cis*-regulatory region on two fitness components, developmental time and productivity.

Materials and methods

Chimeric *Amy* genes

Chimeric *Amy* genes were constructed based on *Amy[1]* and *Amy[6]* alleles, which originated from the *Amy-distal* gene of the KO140 and 1420#1 strains of *D. melanogaster*, respectively (see Inomata *et al*, 1995b). The chimeric *Amy* gene consisted of three regions: 616 bp of 5′-flanking region (*cis*-regulatory region), 1482 bp of coding region and 260 bp of 3′-flanking region. Its structure is shown in Figure 1a. There were 16 nucleotide differences in the 5′-flanking region between the *Amy[1]* and *Amy[6]* alleles. In all, 21 nucleotide differences, resulting in five amino-acid changes, occurred in the coding region. First, each gene region was amplified by the PCR method using the primers and *Amy[1]* or *Amy[6]* gene as a template DNA. The PCR primers used were as follows; Notd−600,

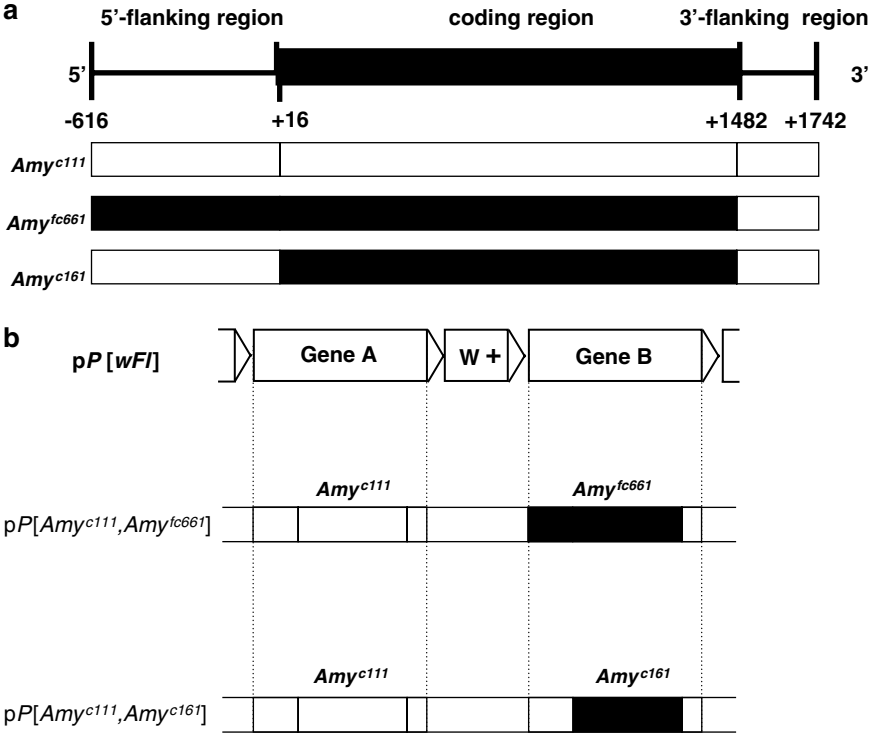

Figure 1 (**a**) Structure of the chimeric *Amy* genes. An adenine of the first codon (Met) was numbered as +1. Numbers, −616, +16, +1482 and +1742, indicate nucleotide position at which *Not*I, *Spe*I, *Sma*I and *Eco*RI restriction sites were introduced, respectively. Top: Horizontal lines indicate 5′- and 3′-flanking regions and black bar indicates coding region. White and black boxes represent the region originated from *Amy[1]* and *Amy[6]* genes, respectively. (**b**) Structure of two transformation constructs, p*P*[*wfl:Amy[c111]*, *Amy[fc661]*] and p*P*[*wfl:Amy[c111]*, *Amy[c161]*], used in this study. The chimeric *Amy* genes were tandemly inserted into the p*P*[*wfl*] vector. Gene A indicates the *Amy[c111]* gene in both the *Amy[fc661]* and *Amy[c161]* strains. Gene B indicates the *Amy[fc661]* and *Amy[c161]* genes in the *Amy[fc661]* and *Amy[c161]* strains, respectively. w+ indicates mini-*white* gene in the vector. White and black boxes represent the region originated from *Amy[1]* and *Amy[6]* genes, respectively.

5′-CGGGCGGCCGCTGTAGCGTGAGATTCCTAA-3′ and Sped+, 5′-GTCACTAGTGTGCCTCGCCCT-3′ for amplification of the 5′-flanking region, Sped−, 5′-CCGAC TAGTGACTTGGCCAGAAACATG-3′ and Sma+1500, 5′-TCCCCCGGGACAACTGGGGCAAAGACC-3′ for amplification of the coding region, and Sma+1485, 5′-TCCCCCGGGTTACAACTTGGCGTTGAC-3′ and Eco+1730, 5′-CCGGAATTCATCACGCTGAATACCGAG-3′ for amplification of the 3′-flanking region. Each primer contains a restriction enzyme site that enables us to make any combinations of Amy^1- or Amy^6-originated regions in the 5′-flanking, coding and 3′-flanking regions. *Not*I, *Spe*I, *Sma*I and *EcoR*I restriction sites were introduced at nucleotide positions −616, +16, +1482 and +1742, respectively (the numbering scheme starts at +1 at the adenine of the first codon (Met)). Note that introduction of the *SpeI* site results in one amino-acid replacement (Ile to Leu) at the seventh amino-acid position. It is considered that this replacement has no effect on AMY activity, since it is located in the region coding for signal peptide (Boer and Hickey, 1986). The PCR fragments were subcloned into pGEM T-easy cloning vector (Promega), and then sequenced to check for PCR errors. Each region was combined with restriction enzyme sites, and three chimeric *Amy* genes, Amy^{c111}, Amy^{c161} and Amy^{fc661}, were constructed (Figure 1a). The Amy^{c111} gene contains *cis*-regulatory, coding and 3′-flanking regions originated from the Amy^1 gene. Similarly, the Amy^{c161} gene contains coding region originated from the Amy^6 gene and the other regions from the Amy^1 gene. The Amy^{fc661} gene contains 3′-flanking region originated from the Amy^1 gene and the other regions from the Amy^6 gene.

Plasmid constructs

For *P*-element-mediated germline transformation of *D. melanogaster*, chimeric *Amy* genes were introduced into the p*P*[*wFl*] vector (Siegal and Hartl, 1996). It is well known that expression among transformants of the same construct differs according to its genomic position, the so-called position effect (Spradling and Rubin, 1983; Laurie-Ahlberg and Stam, 1987; Brennan and Dickinson, 1988; Wilson *et al*, 1990). However, we overcame the problem by introducing two chimeric *Amy* genes into the *p*P[*wFl*] vector (see Figure 1b) to simultaneously insert the two constructs into a particular genomic position. In this study, the Amy^{c111} gene was used as a genomic-position control gene, and the Amy^{c161} or Amy^{fc661} genes were used as test genes. Two transformation constructs, p*P*[*wfl:*Amy^{c111}, Amy^{fc661}] and p*P*[*wfl:*Amy^{c111}, Amy^{c161}], were generated as shown in Figure 1b.

Drosophila transformation and genetic manipulations

P-element-mediated transformation of *D. melanogaster* was carried out using the standard method (Rubin and Spradling, 1982; Spradling and Rubin, 1982) with helper plasmid pπwc (Nitasaka, personal communication) and an isogenic host strain w^{1118}, which was full-sib mated for 14 generations. Hereafter, we denote this host strain as *w-sib14*. Three independent transformants with *P*[*wfl:*Amy^{c111}, Amy^{c161}] insertion and two independent transformants with *P*[*wfl:*Amy^{c111}, Amy^{fc661}] insertion were obtained. Since the *Amy* genes are located on the second chromosome and the host strain has $AMY^{1,3}$ isozymes, the second chromosome of the transformants was finally replaced by the Amy^{null} chromosome. The transformants containing the insertion on the third chromosome were used in this study. To increase the number of independent transformants containing insertion on the third chromosome, the insertion on X and second chromosome were mobilized to new location through genetic cross using the *w; CyO/Sp*; *TM3*, *Ubx/Sb P*[ry^+ Δ2–3](99B) strain as source of transposase (*P*[ry^+ Δ2–3](99B), Robertson *et al*, 1988). Following transformation and mobilization, all strains were crossed to the *w-sib14; c* Amy^{null}*; TM3, Sb, Ser/Dr* strain to exchange genetic background (*w-sib14* for the X and third chromosomes, and *c* Amy^{null} for the second chromosome). This included eight and nine independent lines of the $Amy^{c111,fc661}$ and $Amy^{c111,c161}$ strains, respectively. Hereafter, we call the former and latter the Amy^{fc661} and Amy^{c161} strains, respectively.

Food media

Two different food media, glucose and starch, were used in this study. Glucose medium contains glucose as a carbohydrate source, and starch medium contains starch as a carbohydrate source. The components of food media were 5% ebios (killed yeast) (w/v), 0.6% agar (w/v), 0.4% propionic acid (v/v) and 10% specific carbohydrate source, glucose or starch. Cornmeal medium was used to maintain stocks. Its components were 7% cornmeal (w/v), 10% glucose (w/v), 5% ebios (w/v), 0.6% agar (w/v) and 0.4% propionic acid (w/v).

Sample collection for AMY activity assay

Five male and five female adult flies were transferred to a new vial containing each test food medium, and allowed to lay eggs at 25°C. After 3 days, adults were collected from the vials, and then frozen at −80°C. After three additional days, three third-instar larvae, chosen at random, were collected from the vials without distinguishing sexes, washed with distilled water, and then kept at −80°C. Three replicates of larvae and adults were prepared per line for each test food medium.

Measurement of AMY activity

In this study, AMY activity was represented by the intensity of AMY isozyme bands separated on acrylamide gels. Three larvae and three adult flies were sampled without distinguishing the sexes. They were homogenized with 100 μl of a buffer (pH 8.9) (0.1 M Tris-borate, 5 mM $MgCl_2$ and 10% sucrose (w/v)) by sonication. The homogenates were centrifuged at 10 000 rpm for 5 min and the supernatants were used for assay. To equalize samples applied to the gels, the protein content of each sample was quantified using the commercially available kit, BCA protein assay reagent (Pierce). The procedure essentially followed the manufacturer's protocol. In total, 1 ml of the BCA working reagent made from reagent A (sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartrate in 0.1 N sodium hydroxide) and reagent B (4% copper sulfate solution) in the ratio of 50:1 was added to 25 μl distilled water and 25 μl of the supernatant. After incubation for 30 min at 37°C, the absorbance was measured at 562 nm using spectrophotometer.

The samples with equal protein content (0.52 mg) were 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 4°C under 300 V constant condition, the gel was kept in starch solution (2% soluble starch (w/v), 0.1 M Tris-HCl (pH 7.4) and 20 mM $CaCl_2$) for 30 min at 37°C. The gels were briefly washed with water, and incubated in solution without starch (0.1 M Tris-HCl (pH 7.4) and 20 mM $CaCl_2$) for 30 min at 37°C. Subsequently, they were briefly washed with water, and stained in I_2–KI solution. Gel images were captured into computer by a scanner, and then the band intensity of AMY¹ and AMY⁶ isozymes on a gel was measured, as an index of AMY isozyme activity, using NIH image software (http://rsb.info.nih.gov/nih-image/).

Measurement of the fitness components

Five virgin females and five males from the cornmeal food vials were crossed on starch food. Three virgin females and three males from the cornmeal food were crossed on glucose food. When the parental flies died, they were replaced by flies of the same condition. After 3 days, the parents were discarded. The developmental time index was obtained by calculating the average number of days of eclosion of F1 progeny since the time of crosses. Productivity was calculated as the ratio of the number of progeny to parent input flies. Counting for developmental time and productivity was carried out from the 9th day to the 17th day for both food media.

Data analysis

Statistical analysis was carried out using the StatView software version 4.5. Three replicates of AMY activity and fitness components for each line were used in analyses.

The following two comparisons were performed by two-way analysis of variance (ANOVA). First, to evaluate effect of polymorphism in both 5′-flanking (*cis*-regulatory) and coding regions, activity of the AMY⁶ isozyme produced by the chimeric Amy^{fc661} gene was compared with that of AMY¹ isozyme produced by the chimeric Amy^{c111} gene in the eight Amy^{fc661} strains. Second, to evaluate effect of polymorphism in coding region, activity of the AMY⁶ isozyme produced by the chimeric Amy^{c161} gene was compared with that of AMY¹ isozyme produced by the chimeric Amy^{c111} gene in the nine Amy^{c161} strains. In these comparisons, the position effect of transgenes is canceled out or minimized, since the two genes under comparison have the same position in the genome. The ANOVA model was as follows, $Y_{ijk} = u + A_i + L_j + (A^*L)_{ij} + e_{ijk}$, where Y is AMY activity, u was the overall mean, A_i is the ith *Amy* allele effect (either Amy^1 or Amy^6), L_j is the jth independent transformant line effect in the strain ($j = 1$–8 or 1–9), $(A^*L)_{ij}$ is their interaction effect and e_{ijk} is the error term. All effects were considered random except for the allele effect. The sum of the AMY¹ and AMY⁶ activity was regarded as total AMY activity. The difference in the total AMY activity between the Amy^{fc661} and Amy^{c161} strains should reflect effect of polymorphism in the 5′-flanking (*cis*-regulatory) region between the chimeric Amy^{fc661} and Amy^{c161} genes. The total AMY activity was analyzed by the nested ANOVA. The model of the nested ANOVA was as follows, $Y_{ijk} = u + S_i + B_{ij} + e_{ijk}$, where Y is total AMY activity, u is the overall mean, S_i is the ith transformant strain effect (either Amy^{fc661} or Amy^{c161} strain), B_{ij} was the random contribution for the jth independent transformant line ($j = 1$–8 or 1–9) within the ith transformant strain and e_{ijk} is the error term. The strain effect was considered as the fixed effect. As in the case of the total AMY activity, the difference in fitness between the Amy^{fc661} and Amy^{c161} strains should reflect effect of polymorphism in the 5′-flanking (*cis*-regulatory) region between the chimeric Amy^{fc661} and Amy^{c161} genes. Fitness components data were analyzed by the nested ANOVA. The model of the nested ANOVA was as follows, $Y_{ijk} = u + S_i + B_{ij} + e_{ijk}$, where Y is fitness component, u is the overall mean, S_i is the ith transformant strain effect (either Amy^{fc661} or Amy^{c161} strain), B_{ij} is the random contribution for the jth independent transformant line ($j = 1$–8 or 1–9) within the ith transformant strain and e_{ijk} is the error term. The strain effect was considered to be a fixed effect.

Results

Effect of polymorphism in both the 5′-flanking and coding regions on the AMY activity

To assess the effect of polymorphism on the AMY activity in both 5′-flanking and coding regions, we compared AMY¹ activity with AMY⁶ activity in the eight Amy^{fc661}

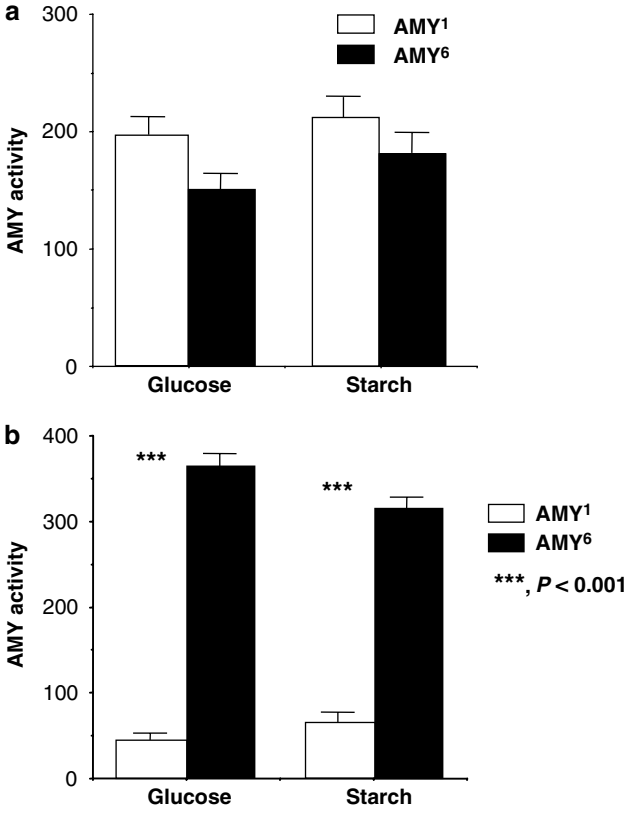

Figure 2 Effect of polymorphism on AMY activity of the 5′-flanking and coding regions in larvae (**a**) and adults (**b**) on glucose and starch media. White and black bars indicate average AMY¹ and AMY⁶ activity, respectively, with standard error in the Amy^{fc661} strain. *** Indicates $P < 0.001$. AMY activity was represented by the band intensity of the AMY isozyme separated on acrylamide gels.

strains on the glucose and starch media at the larval and adult stages (Figure 2). Compared with the AMY^1 activity, the AMY^6 activity was slightly lower at the larval stage, but the difference was not significant on both media ($F_{1,32}=1.844$, $P=0.1840$ and $F_{1,32}=0.284$, $P=0.5980$ on the glucose and the starch media, respectively, see Figure 2a). At the adult stage, on the other hand, the AMY^6 activity was significantly higher than the AMY^1 activity on both media ($F_{1,32}=23.655$, $P<0.0001$ and $F_{1,32}=26.505$, $P<0.0001$ on the glucose and the starch media, respectively, see Figure 2b). Other effects were not significant at 5% level in either media at both stages. These results indicate that the effect of polymorphism on AMY activity in both the 5′-flanking and coding regions is different between the two stages.

Effect of polymorphism in the coding region on the AMY activity

To evaluate the effect of polymorphism in the coding region on the AMY activity, we compared the AMY^6 activity with the AMY^1 activity in the nine *Amy^{c161}* strains on the glucose and starch media at the larval and adult stages (Figure 3). In both media, the AMY^6 activity was significantly lower than the AMY^1 activity at the larval stage ($F_{1,35}=77.684$, $P<0.0001$ and $F_{1,36}=54.461$, $P<0.0001$ on the glucose and the starch media, respectively, see Figure 3a), while the difference was not significant at the adult stage on both media ($F_{1,36}=0.209$, $P=0.6503$ and $F_{1,36}=0.075$, $P=0.7851$ on the glucose and the starch media, respectively, see Figure 3b). In larvae, the line effect and interaction effect between line and *Amy* allele were also significant in both media (line effect: $F_{8,35}=17.484$, $P<0.0001$ and $F_{8,36}=7.912$, $P<0.0001$ on the glucose and the starch media, respectively, and interaction effect: $F_{8,35}=5.455$, $P=0.0002$ and $F_{8,36}=3.446$, $P=0.0048$ on the glucose and the starch media, respectively). In adults, the line effect was significant in glucose medium ($F_{8,36}=2.231$, $P=0.0479$). Other effects were not significant at the 5% level. These results indicate that the effect of the 21 nucleotide differences in the coding region is different between the two stages, even though the same isozymes are encoded.

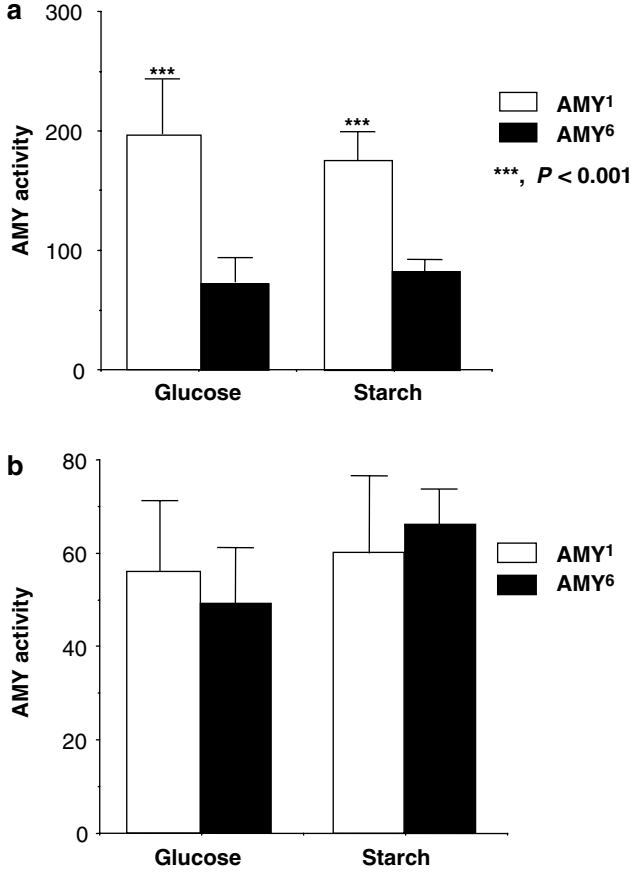

Figure 3 Effect of polymorphism on AMY activity of the coding region in larvae (**a**) and adults (**b**) on glucose and starch media. White and black bars indicate average AMY^1 and AMY^6 activity, respectively, with standard error in the *Amy^{c161}* strain. *** Indicates $P<0.001$. AMY activity was represented by the band intensity of the AMY isozyme separated on acrylamide gels.

Effect of polymorphism in the 5′-flanking region on AMY activity

To evaluate the effect of polymorphism in the 5′-flanking (*cis*-regulatory) region on the AMY activity, we compared the total AMY activity, which is the sum of AMY^1 and AMY^6 activity, between the *Amy^{fc661}* and *Amy^{c161}* strains on the glucose and starch media at the larval and adult stages (Figure 4). At the larval stage, total AMY activity

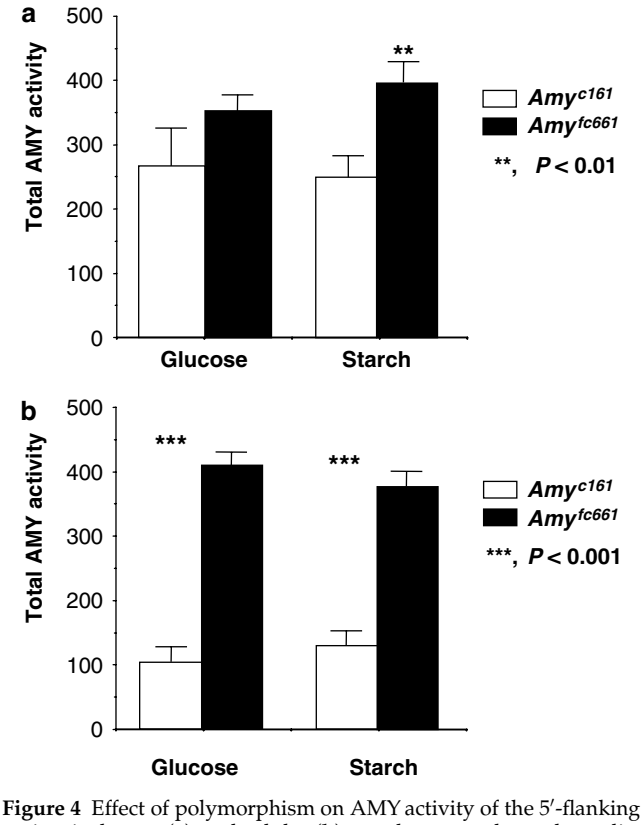

Figure 4 Effect of polymorphism on AMY activity of the 5′-flanking region in larvae (**a**) and adults (**b**) on glucose and starch media. White and black bars indicate average AMY activity in the *Amy^{c161}* and *Amy^{fc661}* strains, respectively, with standard error. ** and *** indicate $P<0.01$ and $P<0.001$, respectively. AMY activity was represented by the band intensity of the AMY isozymes separated on acrylamide gels.

in the Amy^{fc661} strain was significantly higher than that in the Amy^{c161} strain on the starch medium ($Fs_{1,15}=9.590$, $P<0.01$, see Figure 4a). At the adult stage, total AMY activity in the Amy^{fc661} strain was significantly higher than that in the Amy^{c161} strain on both the glucose and starch media ($Fs_{1,15}=82.983$, $P<0.001$ and $Fs_{1,15}=67.863$, $P<0.001$ on the glucose and the starch media, respectively, see Figure 4b). Other effects were not significant at the 5% level. These results indicate that 16 nucleotide differences in the *cis*-regulatory region have effect on the AMY activity between the two allelic classes.

Effect of polymorphism in *cis*-regulatory region on fitness components

Significant effect of polymorphism in the *cis*-regulatory region on AMY activity was found. Therefore, if the effect is sufficiently large, it may be possible to detect the effect on fitness. To test whether the difference in *cis*-regulatory elements causes the difference in fitness, we measured two fitness components, developmental time and productivity, on the glucose and starch media in the Amy^{fc661} and Amy^{c161} strains. Average developmental time was 13.21 and 13.13 days on glucose media in the Amy^{fc661} and Amy^{c161} strains, respectively. On the starch media, average developmental time was 12.89 and 12.72 days in the Amy^{fc661} and Amy^{c161} strains, respectively (see Figure 5a). No significant effect on developmental time was found between the Amy^{fc661} and Amy^{c161} strains on both media at 5% level ($Fs_{1,15}=0.155$ and $Fs_{1,15}=0.206$ on the glucose and the starch media, respectively). Average productivity was 4.93 and 4.16 individuals on the glucose media in the Amy^{c161} and Amy^{fc661} strains, respectively. On the starch media, average productivity was 2.16 and 5.07 individuals in the Amy^{c161} and Amy^{fc661} strains, respectively (see Figure 5b). We found 2.35 times difference in productivity between the Amy^{fc661} and Amy^{c161} strains on the starch media at the face value. However, there was no significant effect on developmental time between the Amy^{fc661} and Amy^{c161} strains on both media at the 5% level ($Fs_{1,15}=0.188$ and $Fs_{1,15}=0.240$ on the glucose and the starch media, respectively). Significant effect was found between the lines for each strain ($Fs_{15,34}=2.2751$, $P<0.05$ and $Fs_{15,34}=9.021$, $P<0.001$ on the glucose and the starch media, respectively).

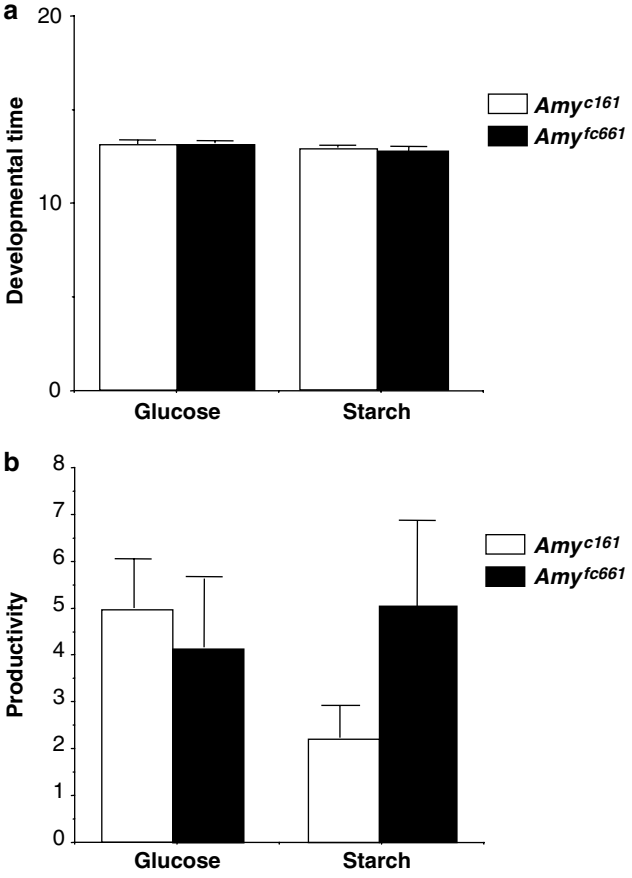

Figure 5 Effect of polymorphism on two fitness components, developmental time (**a**) and productivity (**b**) on glucose and starch media. White and black bars indicate average fitness components in the Amy^{c161} and Amy^{fc661} strains, respectively, with standard error.

Discussion

Effect of polymorphism on AMY activity

The effects of the coding region variants on AMY activity were different between the larvae and adults; similarly, the combined effect of the 5′-flanking and coding regions differed between larvae and adults. On the other hand, the effect of polymorphism in the 5′-flanking region alone did not differ between the two stages. In larvae, it is likely that higher AMY activity caused by the 5′-flanking sequence derived from the Amy^{6} allele (see Figure 4a), whereas lower AMY activity was caused by the coding sequence derived from the Amy^{6} allele (see Figure 3a). The combined result was, therefore, no significant difference in AMY activity (see Figure 2a). On the other hand, in adults the higher AMY^{6} activity caused by the combination of 5′-flanking and coding sequences (see Figure 2b) is most likely to be due to the 5′-flanking sequence (see Figure 4b), because no significant effect on AMY activity was found between the coding sequences of the two alleles (see Figure 3b). If this is true, our results suggest that effect of polymorphism in the coding region is different between larvae and adults, even though the same AMY isozymes are encoded. In other words, AMY isozymes show stage-specific activity. One plausible explanation for this observation is that the post-transcriptional processing of the mature protein, such as the stability of protein and translational efficiency, may differ between larvae and adults.

In the Amy^{c161} strain, AMY^{1} activity was significantly higher than AMY^{6} activity in larvae in both glucose and starch media (see Figure 3a), indicating that the coding sequence of the Amy^{1} allele results in higher AMY activity than that of the Amy^{6} allele. As both the genetic background and the *cis*-regulatory region is the same for the chimeric genes (Amy^{c111} and Amy^{c161}), the amount of mRNA would be expected to be the same. Therefore, the difference in AMY activity seems to be due to the post-transcriptional differences caused by 21 nucleotide differences in the coding region of the two allelic classes. Five of these differences are nonsynonymous and may cause the difference in catalytic efficiency. Stability of mRNA, stability of protein and translational efficiency may also be affected by synonymous differences.

The 5′-flanking sequence derived from the Amy^6 allele always causes higher AMY activity than that from the Amy^1 allele. This result indicates that the Amy^6-derived 5′-flanking sequence produces more mRNA, because the level of AMY activity correlates with the amount of mRNA (Hickey, 1981; Yamate and Yamazaki, 1999). There were only 16 nucleotide differences in the 5′-flanking region between the two alleles. No nucleotide differences were located in the core sequence of the putative *cis*-regulatory elements. A single-nucleotide difference may have a major effect on the level of the *Amy* gene expression, or alternatively, the difference in the mRNA level may be caused by nonrandom associations between polymorphism at different sites.

Since a highly inbred laboratory strain was used as a host, and assays were performed with only two allelic classes, our results might not be easily extended to other isozymes. Similarly, they might not mirror the effects of polymorphism in variable genetic backgrounds and/or under natural conditions. However, our results clearly indicate that nucleotide polymorphism in the 5′-flanking and coding regions appear to produce different AMY activity.

Effect of polymorphism on fitness

Yamazaki and Matsuo (1984) and Matsuo and Yamazaki (1984) showed that fitness was positively correlated with the inducibility (response ability to different food media) of AMY activity but not with activity itself. Therefore, they proposed that variation of all regulatory factors including transcriptional and post-transcriptional factors are important for adaptive evolution. The adaptive significance of changes in regulatory factors has been pointed out by several authors (eg King and Wilson, 1975). Recently, Wittkopp, Haerum and Clark (2004) indicated that *cis*-acting regulatory changes are more important than *trans*-acting ones for interspecific expression differences. In this study, we measured two fitness components, developmental time and productivity, to examine the effect of *cis*-acting regulatory changes. No significant effect was detected. However, on the starch media, the Amy^{fc661} strain showed 2.35 times more productivity than the Amy^{c161} strain. The difference of such magnitude may be biologically significant. Our design may have had insufficient statistical power to obtain a statistically significant result because of the small number of transformants examined.

Evolutionary implications

Some models of evolution of multigene families assume that the ancestral gene has two or more distinct but pleiotropically constrained functions (Piatigorsky and Wistow, 1991; Hughes, 1994), while other models are free of such an assumption (eg Ohno, 1970). Our results suggest that the relative AMY activity encoded by certain *Amy* alleles differs among the developmental stages, which indicates that they may have distinct subfunctions in *Drosophila*. This observation supports the model proposed by Piatigorsky and Wistow (1991) and Hughes (1994) who suggested that genes with multiple subfunctions can be important in the evolution of at least some multigene families.

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