Genet. Res. Camb. (1992), **59**, *pp.* 85–92 *With 4 text-figures Printed in Great Britain*

Multiple amylase genes in *Drosophila ananassae* and related species

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(*Received 1 May 1991 and in revised form 9 December 1991*)

Summary

The number and organization of amylase genes in *Drosophila ananassae* were investigated through classical genetic methods and *in situ* and filter hybridizations. At least four genes may be active in *D. ananassae*, organized as two independent pairs of closely linked copies on the 2L and 3L chromosomal arms. Several other species of the *D. ananassae* subgroup were studied and show the same chromosomal locations, suggesting an ancient duplication event. However, the number of *Amy* copies seems to be higher in the *D. ananassae* multigene family, and there is a striking intraspecific molecular differentiation.

1. Introduction

Many cases of multiple amylase gene loci have been reported in the Arthropoda. In the Crustacea, two active loci were found in *Sphaeroma* (Laulier, 1988), and six loci in *Asellus aquaticus*, five of which were polymorphic (Oxford, 1986). In the Insecta, two linked loci were reported in *Bombyx mori* (Kikkawa, 1953), *Musca domestica* (Ogita, 1968) and in several species of *Tribolium* (Pope *et al.*, 1986). Within the Drosophilidae, an *Amy* gene duplication was first shown by genetic analysis in *Drosophila melanogaster* (Bahn, 1967), and more recently it has been confirmed at the molecular level that the duplication is general within the species *D. melanogaster* (Levy *et al.*, 1985; Boer & Hickey, 1986; Doane *et al.*, 1987; Langley *et al.*, 1988). The duplicated gene structure occurs throughout the *D. melanogaster* species subgroup (Dainou *et al.*, 1987; Payant *et al.*, 1988) and also has been reported from several species of the *D. obscura* group (Doane & Norman, 1985; Doane *et al.*, 1987).

We have focused our attention on the widespread tropical species *Drosophila ananassae* (Da Lage *et al.*, 1989). The observation of multibanded electrophoretic phenotypes suggested the presence of multiple amylase gene loci. A marked geographical pattern is observed, with high polymorphism in African populations and near-monomorphism in Pacific populations, in which single-banded patterns are common.

The aim of the present study is to determine the number and structure of the amylase genes in *D. ananassae* and in several related species, using different methods: Mendelian crosses, *in situ* chromosomal hybridizations, and genomic DNA analysis by Southern blotting.

2. Materials and Methods

(i) *Drosophila strains*

D. ananassae strains were collected from various parts of the Tropics. The following strains were made homogeneous for various amylase patterns by inbreeding, and then used in genetic recombination experiments. Taï 13-1610 was extracted from an African population (Ivory Coast) and homogenized for the AMY-1,2,3,4 phenotype, expressed in both adult flies and larvae. Taka 5 was an isofemale line from Takapoto (French Polynesia), expressing only AMY-3 in adults. T 404, homogeneous for AMY-4 in adult flies, was extracted from another isofemale line from Takapoto. We considered these strains to be homozygous for the expressed electromorphs.

For *in situ* hybridizations, we used individuals of *D. ananassae* from Martinique (West Indies), Taï (Ivory Coast), Varanasi (India), Hawaii and Thaïland. This analysis was also extended to other species belonging to the *D. ananassae* subgroup, i.e. *D. malerkotliana* (Madagascar), *D. bipectinata* (New Caledonia), *D. varians* (Philippines). For Southern blotting experiments, we used *D. ananassae* from homozygous lines, namely Taï 13-1610, T 404 and the strain 371-1 from the Bowling Green Drosophila Stock Center, which expresses a constant AMY-3,4 phenotype. *D. varians*

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(Philippines), *D. monieri* (Moorea Is.), *D. parabipectinata* (Mauritius), *D. malerkotliana* (Madagascar) and *D. ercepeae* (Réunion Is.) were also assayed by Southern blotting.

(ii) *Amylase electrophoresis*

Flies were reared on standard sugar-maize medium and fed with a killed-yeast-cornmeal medium prior to gel analysis. This medium increases enzyme production. Amylase electrophoresis was carried out as described by Da Lage *et al.* (1989).

(iii) *DNA probes*

The plasmid pOR-M7, kindly supplied by Dr D. A. Hickey (Ottawa University), was used as a probe (Benkel *et al.*, 1987). It consists of a pUC plasmid containing the *Amy* gene cDNA of *D. melanogaster*. For *in situ* hybridizations, the entire plasmid was labeled by nick-translation with biotin-16-dUTP (Boehringer). For Southern blots, only the amylase cDNA insert (1500 bp) was nick-translated with ^{32}P-labelled deoxynucleotides.

(iv) In situ *hybridizations*

Third instar larvae were dissected in 45% acetic acid, and salivary glands were incubated for three minutes in the fixing solution (one part lactic acid, two parts water, three parts acetic acid) before squashing. Pretreatment and hybridization were adapted from Engels *et al.* (1986). The probe solution was as follows: 1 μg of denatured DNA in 19 μl H_2O, mixed with 10 μl of 20 × SSC, 10 μl of 50% dextran sulphate and 21 μl of formamide. Ten microliters were used per slide. These conditions (35% formamide) are suitable for heterologous probes. Finally, chromosomes were stained with Giemsa.

(v) *Southern blot analysis*

Genomic DNAs were prepared as follows. About 300 mg of flies were homogenized in 5 ml of a solution of 0·1 M-Tris-HCl pH 9; 0·1 M-EDTA. An equal volume of 2% SDS, prewarmed at 65°, was added. The homogenate was incubated for 1 hr at 65°, then centrifuged briefly to remove solid debris. Proteins were precipitated with 8 M potassium acetate (130 μl/ml); the supernatant was RNase- and proteinase K-treated, and phenol-chloroform extracted. DNA was recovered by isopropanol precipitation followed by ethanol-NaCl precipitation. DNA pellets were dissolved in TE.

For each sample, about 3 μg of DNA were digested overnight in 100 μl reactions with a large excess of restriction enzymes in the recommended buffer and in the presence of 100 μg/ml BSA. Digested samples were precipitated and loaded in horizontal 0·7% agarose gels in TBE buffer (Maniatis *et al.*, 1982). After migration, DNA was transferred onto Biodyne nylon membranes by the usual transfer technique of Southern. Filters were hybridized as described in Anderson & Young (1985) at 65° in the presence of 10% dextran sulfate. Washes were carried out with a decreasing ionic force (from 300 mM- to 25 mM-NaCl) at 65–68°.

3. Results

(i) *Crosses between homozygous strains*

Considering that the alpha-amylase protein is a monomer (Kikkawa, 1964; Doane *et al.*, 1975), the existence of a strain homozygous for *Amy 1,2,3,4* is the first evidence for a quadruplicated gene. Crosses using this strain and one-banded individuals should help us to understand the structure of the genes.

Six females of the strain Taï 13-1610 (*Amy 1,2,3,4/Amy 1,2,3,4*) were crossed with males from T 404 (*Amy 4/Amy 4*). All F_1 individuals from the six crosses (F_1-1 to F_1-6) exhibited a AMY-1,2,3,4 pattern (not shown). The F_2 was bred from a mass culture of F_1-1 flies and four phenotypic classes were found: AMY-4, AMY-3,4, AY-1,2,4 and AMY-1,2,3,4 (Table 1). There was no significant difference between males and females for phenotypic frequencies (data not shown). Several backcross pairs were designed as follows: one pair ♀ T 404 × ♂ F_1-1 (BC1), one pair ♀ T404 × ♂ F_{1-2} (BC2), 3 pairs ♀ T 404 × ♂ F_1-3 (BC3) and one pair ♀ F_1 × ♂ T 404 (BC'1). The same four phenotypes as in the F_2 were found in the offspring (Table 1).

The data show that *Amy 1* and *Amy 2* always segregated together, suggesting that the respective coding sequences are closely linked. The results are similar to classical dihybridism, in which the proportions are 9:3:3:1 in the F_2 and 1:1:1:1 in the backcross. Table 1 shows that the observed numbers are in close agreement with Mendelian proportions. The χ^2 values are not significant. According to this dihybridism hypothesis, the genetic structure shown on Fig. 1 is proposed. In the Taï 13-1610 line (*Amy 1,2,3,4/Amy 1,2,3,4*) there would be two independant pairs of active, closely linked, *Amy* copies. We assume that AMY-3 and AMY-4 phenotypes (respectively Taka 5 and T 404 strains) are due to a single active copy each.

To check this hypothesis, another cross was carried out using the *Amy 3/Amy 3* line (Taka 5), instead of the *Amy 4/Amy 4* line. Only one pair yielded numerous offspring. The results are consistent with the predictions of our hypothesis. The F_1 was homogeneous for AMY-1,2,3,4. The four F_2 and backcross phenotypic classes expected, AMY-3, AMY-3,4, AMY-1,2,3, AMY-1,2,3,4, were found with the expected proportions (Table 2). χ^2 values of the hypotheses

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Table 1. *Segregation analysis of isoamylases in F_2 and backcrosses of the cross* ♀ Amy 1,2,3,4/Amy 1,2,3,4 (*Taï 13-1610*) × ♂ Amy 4/Amy 4 (*T 404*). *Numbers of individuals of AMY phenotypes in the F_2 and backcrosses are given, as well as the results of χ^2 tests on the data*

F_2	AMY 4	AMY 3,4	AMY 1,2,4	AMY 1,2,3,4	Total	Test hypothesis 9:3:3:1 3 D.F.
	8	38	33	139	218	$\chi^2 = 6{\cdot}23$
Back cross	AMY 4	AMY 3,4	AMY 1,2,4	AMY 1,2,3,4	Total	Test hypothesis 1:1:1:1 3 D.F. χ^2
BC'1	46	57	49	39	191	3·49
BC1	18	33	28	22	101	5·18
BC2	19	27	12	18	76	6·00
BC3-1	26	33	29	29	117	0·85
BC3-2	41	33	24	32	130	4·46
BC3-3	47	35	26	48	156	8·46*
BC3-4	32	26	30	39	127	2·79
Total	229	244	198	227	898	4·94

χ^2 for homogeneity (backcrosses) is 26·3 (18 D.F.).
The value is not significant at the 5% level. * $P < 0{\cdot}05$.

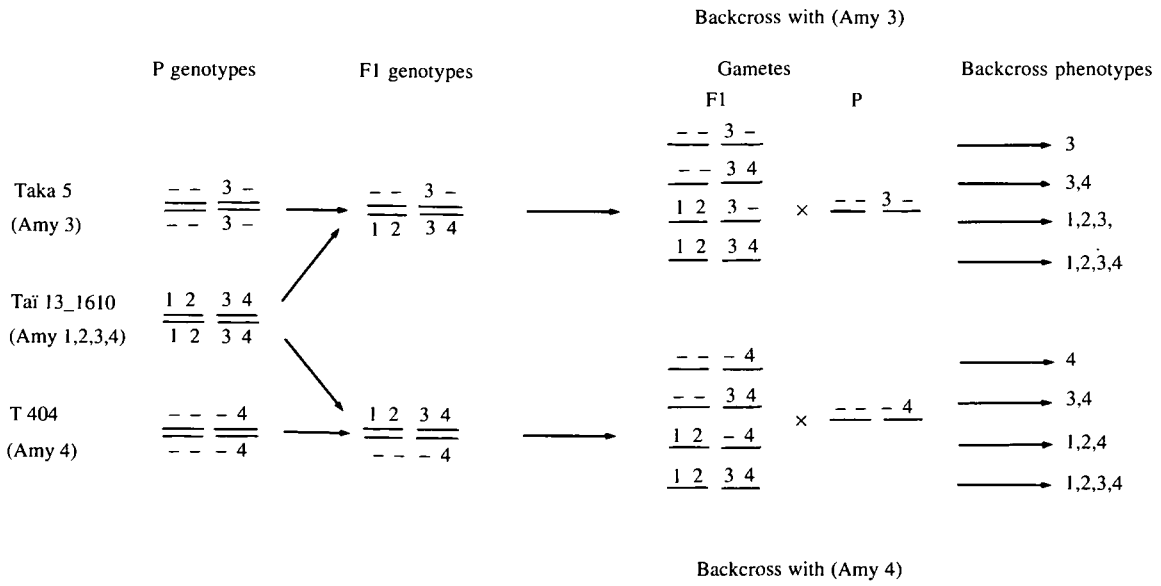

Fig. 1. Likely genetic structure of *Amy* loci in *D. ananassae*, assumed from recombination experiments: two independant pairs of closely linked copies. At each locus, a null allele is inferred to explain the AMY-3 and AMY-4 phenotypes. Crossing Taï 13-1610 with each of these one-banded strains gives, in F_1, four types of equiprobable gametes, corresponding to the observed backcross phenotypic classes.

9:3:3:1 for the F_2 and 1:1:1:1 for the backcross are not significant.

If the two loci *Amy 3* and *Amy 4* are very close to each other, as it is assumed, we expect that a cross *Amy 4/Amy 4* × *Amy 3/Amy 3* (i.e. T 404 × Taka 5) should give results similar to a simple monohybrid cross, with 100% AMY-3,4 F_1 individuals, and proportions 1:2:1 in the F_2 and 1:1 in the backcross. Eventual AMY-null phenotypes would indicate recombination between the two loci. The four crosses were performed and Tables 3 and 4 show that the F_2 and backcross proportions are consistent with the hypotheses except in two of the backcrosses, where a significant excess of heterozygotes was observed. No AMY-null phenotype was recovered.

In addition, we also performed crosses between flies *Amy 3,4/Amy 3,4* and flies *Amy 3,5/Amy 3,5*. The results (not shown) indicated that *Amy 5* was a likely allele of *Amy 4*. Figure 2 is an attempt to place different electromorphs at their respective loci.

(ii) *In situ hybridizations*

The likely genetic structure described above, two genetically independant pairs of copies, is consistent with *in situ* hybridizations. Control analyses in *D.*

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Table 2. *Segregation analysis in F_2 of cross* ♀ Amy 3/Amy 3 (*Taka 5*) × ♂ Amy 1,2,3,4/Amy 1,2,3,4 (*Taï 13-1610*) *and backcross* (*3 pairs* ♀ Amy 3/Amy 3 × ♂ F_1)

F_2	AMY 3	AMY 3,4	AMY 1,2,3	AMY 1,2,3,4	Total	Test hypothesis 9:3:3:1 3 D.F. $\chi^2 = 6{\cdot}83$
	14	65	41	144	264	
Back cross	AMY 3	AMY 3,4	AMY 1,2,3	AMY 1,2,3,4	Total	Test hypothesis 1:1:1:1 3 D.F. χ^2
BC1	7	13	11	9	40	2·00
BC2	10	16	10	15	51	2·41
BC3	19	25	28	27	99	1·96
Total	36	54	49	51	190	3·98

χ^2 for homogeneity (backcrosses) is 2·20 (6 D.F.), not significant at the 5% level.

Table 3. *Segregation analysis of F_2 of cross* ♀ Amy 3/Amy 3 (*Taka 5*) × ♂ Amy 4/Amy 4 (*T 404*)

F_2	AMY 3	AMY 3,4	AMY 4	Total	Test hypothesis 1:2:1 2 D.F. χ^2
F2-1	13	49	28	90	5·71
F2-2	30	70	27	127	1·47
F2-3	30	77	26	133	3·56
F2-4	24	50	28	102	0·35
Total	97	246	109	452	4·17

χ^2 for homogeneity is 11·05 (6 D.F.), not significant at the 5% level.

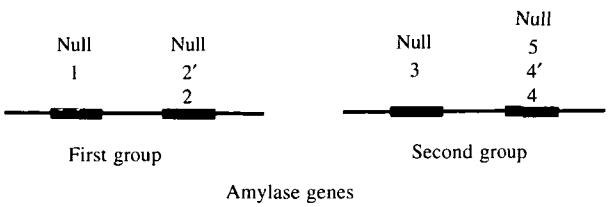

Fig. 2. Hypothetical relationships between some electromorphs and the four structural *Amy* loci. The two pairs *Amy 1*, *Amy 2*, and *Amy 3*, *Amy 4* are placed as in fig. 1. *Amy 2′* may be an allele of *Amy 2*. It coexists with *Amy 2* in African populations, where AMY-1,2,3,4 and AMY-1,2′,3,4 phenotypes are found. In Hawaii, AMY-4 is replaced by a slower isoamylase, named AMY-4′, whose coding gene is likely to be an allele of *Amy 4*. *Amy 5* also proved to be an allele of *Amy 4* (see text). In addition, considering AMY-3 and AMY-4 phenotypes, we assume the possibility of null alleles at each locus.

Table 4. *Backcrosses* ♀ Amy 3/Amy 3 (*Taka 5*) × ♂ *F_1-1 from Taka 5 × T 404*

Back cross	AMY 3	AMY 3,4	Total	Test hypothesis 1:1 1 D.F. χ^2
BC 1	39	29	68	1·47
BC 2	53	39	92	2·13
BC 3	47	50	97	0·09
BC 4	67	58	125	0·65
BC 5	33	63	96	9·37**
BC 6	19	56	75	18·25**
BC 7	43	56	99	1·71

χ^2 for homogeneity is 33·7 (6 D.F.), significant at the 1% level. Data cannot be pooled. ** $P < 0{\cdot}01$.

melanogaster (not shown) consistently showed a single hybridization region on the 2R chromosomal arm, in agreement with Gemmill *et al.* (1985). In *D. ananassae*, two labelled sites were observed (Fig. 3*a*). The first one has been identified on the left arm of second chromosome (2L), within the large cosmopolitan inversion 2LA (Futch, 1966) and on one of the 2LA breakpoints, corresponding to the section 37C in the nomenclatures of Moriwaki and Ito (1969) and Tomimura and Tobari (unpublished). Consequently, depending on the chromosomal arrangement, this *Amy* region may be located in a proximal or distal position. Figure 3*b* shows the proximal arrangement, with an additional, less intensely labelled site, 2L′, which was found with certainty only in the Thaïland strain (Section 39D–40A). The second common site was observed in a basal position on the left arm of the third chromosome (3L), very close to the chromocentre (Section 81C). This location has been found in several geographically distant strains of *D. ananassae*, but not in all of them. However, in some cases, this locus did not easily hybridize to the probe. We certainly cannot conclude that, when the 3L site is unlabelled, the corresponding *Amy* gene is deleted.

Other species of the *D. ananassae* subgroup were studied. *D. varians* belongs to the *ananassae* complex and also shows two labelled sites in chromosomes 2L and 3L, as in *D. ananassae*. This indicates that two independent sets of genes exist in that species. When

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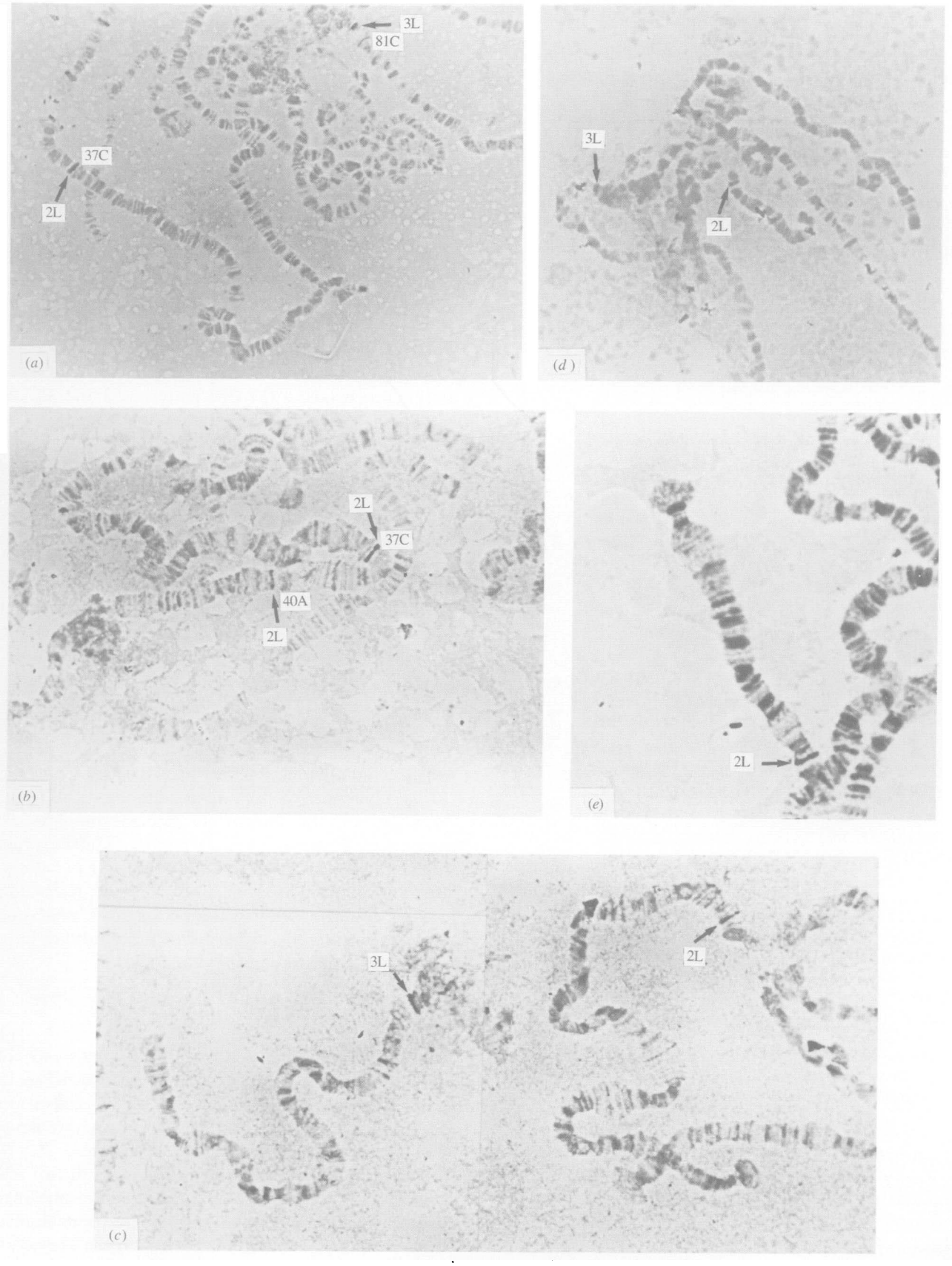

Fig. 3. *In situ* hybridization of polytene chromosomes with the *Amy* cDNA probe of *D. melanogaster*. *Amy* loci are indicated by arrows. (*a*), *D. ananassae* (Hawaii), showing two labelled sites (2L distal, 3L); (*b*), *D. ananassae* (Thailand), showing the 2L chromosome locus in a proximal position and the additional 2L′ signal; (*c*), *D. varians*, showing two signals on 2L and 3L arms; (*d*), *D. bipectinata*, showing signals on 2L and 3L arms; (*e*), *D. malerkotliana*, showing *Amy* locus on the 2L arm.

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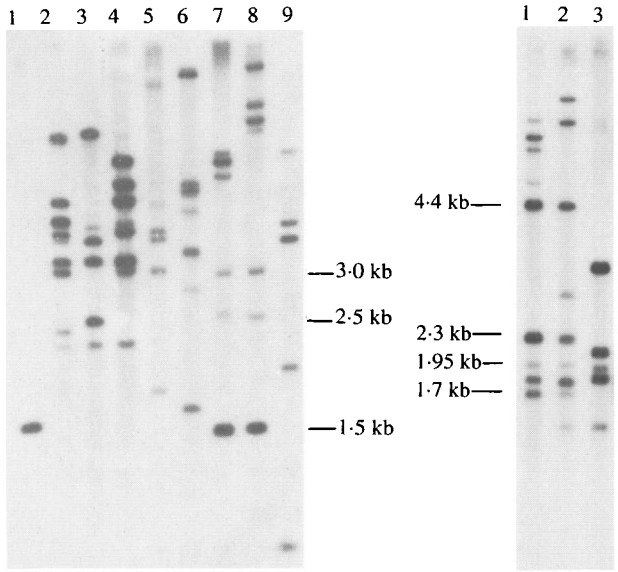
Table 5. *Results of* in situ *hybridizations of the* D. melanogaster Amy *clone pOR-M7 on strains of* Drosophila ananassae *and three related species*

Strain of *D. ananassae*	Chromosomal locus 2L	3L	2L′
Hawaii	+	+	−
Martinique	+	+	?
Cuba	+	+	−
Taï	+	+/−	−
Varanasi	+	+	−
Thaïland	+	−	+
D. varians	+	+	−
D. bipectinata	+	+	−
D. malerkotliana	+	?	−



(*a*) (*b*)

Fig. 4. Restriction patterns of genomic DNA probed with *Amy* cDNA of *D. melanogaster*. (*a*), *Pst*I digestion. Lane 1: *Amy* cDNA from pOR-M7; lane 2: Taï 13-1610 (*D. ananassae*); lane 3: T 404 (*D. ananassae*); lane 4: 371-1 (*D. ananassae*); lane 5: *D. monieri*; lane 6: *D. varians*; lane 7: *D. parabipectinata*; lane 8: *D. malerkotliana*; lane 9: *D. ercepeae*. Sizes of bands common to *D. parabipectinata* and *D. malerkotliana* are indicated. (*b*), *Sal*I digestions of three strains of *D. ananassae*. Lane 1: Taï 13-1610; lane 2: 371-1; lane 3: T 404. Sizes of bands common to Taï 13-1610 and 371-1 are indicated.

compared to *D. ananassae*, the basal 3L position is exactly the same, while on the 2L arm, the spot is closer to the chromocentre (Fig. 3*c*). Two other species, *D. malerkotliana* and *D. bipectinata*, more distantly related to *D. ananassae*, were assayed for *in situ* hybridizations. In both species, a site of strong labelling was found on chromosome 2L. In *D. bipectinata*, a basal 3L site was also observed, as a sharp darker band inside the first dark band (Fig. 3*d*). In *D. malerkotliana*, only the 2L locus was ascertained (Fig. 3*e*), although the other locus, more difficult to detect, could be present. Table 5 summarizes the cytogenetical observations presently available.

(iii) *Southern experiments*

Figure 4 shows the complexity of restriction patterns of *Amy* regions in *D. ananassae* (up to ten bands for a single sample). Estimating the number of gene copies from these data is very difficult since no restriction map can be inferred using the *D. melanogaster* results, but the results are consistent with the existence of several genes, as suggested by the previous experiments. There is a striking molecular differentiation between the AMY-1,2,3,4 line (Taï 13-1610), the AMY-4 line (T 404) and the AMY-3,4 Bowling Green strain (371-1). The three strains share only a few bands in the *Pst*I pattern (2·2 and 3·2 kb) and none in the *Sal*I pattern, although there are more similarities considering only Taï 13-1610 and 371-1. Three other enzymes were assayed with the same strains: *Bam*HI, *Eco*RI, *Hind*III, and the restriction patterns for all of them (not shown) were very different from each others, except for some similarities in *Bam*HI digestions of Taï 13-1610 and 371-1.

The other species studied also show a complex banding pattern, suggesting the occurrence of several gene copies. Figure 4*a* (*Pst*I digestion) shows no close relationship between the different species, except between *D. parabipectinata* and *D. malerkotliana* (three common bands: 3·0 kb, 2·5 kb, 1·5 kb). Several common bands (2·2 kb, 1·9 kb, 0·8 kb, not shown) also appear on the *Bam*HI pattern for theses two species, which are more closely related (Bock & Wheeler, 1972). There is no common band among the different members of the *ananassae* species complex, *D. ananassae*, *D. monieri* and *D. varians*, which express the same isoamylase AMY 3 (Da Lage *et al.*, 1989). However, considering all the different restriction enzyme patterns used in this experiment, *D. ananassae* exhibits consistently more bands, and so may have more *Amy* copies, than the other species.

4. Discussion

Our results provide evidence for the existence of multiple Amylase gene loci in *D. ananassae*, where at least four gene copies appear to be active, or potentially so. So many active *Amy* genes have never been found in species of *Drosophila* before.

The general organization of *Amy* genes in two sets is conserved within *D. ananassae*, since *in situ* labellings remain constant among very distant populations. Both chromosomal loci are present in the Hawaiian strain, which expresses only two electromorphs, as well as in the African line Taï 13-1610, which expresses four electromorphs, so that there might be no clear relationship betwee the number of active genes and the total copy number.

The problem of single-banded phenotypes deserves

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discussion, because it raises the question of the number of active copies, rather than the total number of copies, as has been pointed out (Gemmill *et al.*, 1986; Doane *et al.*, 1987; Langley *et al.*, 1988). For example, *D. varians* apparently harbours several *Amy* gene copies, and chromosomal locations similar to those in *D. ananassae* are found. But in this species, only one electromorph, AMY-3, is expressed (Da Lage *et al.*, 1989). Most likely, one single copy is active, but concerted evolution by gene conversion (Ohta, 1983), leading to several identical genes (or gene products) cannot be excluded. A unique amylase isozyme may be due to the same sequence (or sequences producing the same protein) in several different copies. This is probably the case in *D. erecta*, where partial nucleotide sequences of both copies indicate 100% homology (Bally-Cuif *et al.*, 1990). Hawley *et al.* (1990) have shown by somatic transformation experiments that the two copies in the *D. melanogaster* Oregon-R strain are functional, both expressing AMY-1. On the other hand, some AMY-1 phenotypes in *D. melanogaster* are caused by the inactivation of one copy. This was first shown by Bahn (1967). By crossing AMY-1 and AMY-4,6 individuals, Klarenberg *et al.* (1986) found a few AMY-4 intergenic recombinant phenotypes, which could not appear unless one copy of the AMY-1 parental gene was silent. The strain used in this experiment was different from that used by Hawley *et al.* (1990). Thus, several explanations are possible for single-banded patterns, and further molecular investigations will be necessary to solve the problem in *D. ananassae*.

The data presented above suggest that several duplication events occurred during the evolution of the *D. ananassae* subgroup. The simplest evolutionary scheme would be that a very ancient duplication led to two closely linked copies. Then a second event duplicated this pair of copies on a different chromosome. This could have happened through chromosomal rearrangements, which are known to be frequent in *D. ananassae*. This species of *Drosophila* is the only one where spontaneous male crossing-over is known (Singh, 1985). The basal 3L location is surprising, because crossing over involving the centromeric region is not frequent (Stephan & Langley, 1989). The original locus is not known: neither the 2L or 3L arms are homologous to the 2R arm of *D. melanogaster*, which carries the *Amy* genes in this species (Sturtevant & Novitski, 1941). The second event (translocation) probably took place before the separation between the *D. ananassae* complex and the *D. bipectinata* complex. The consistently higher number of DNA fragments which hybridize to the *Amy* probe in *D. ananassae* suggests that this species may have the highest number of copies. Thus, we may assume that more recent duplications occurred in the *D. ananassae* lineage, leading to more than two copies per chromosomal locus, but we don't know whether the number of copies is the same in both sets of genes. Similar *Amy* gene clusters are known in mammals (Crerar & Rooks, 1987).

Several examples of duplication of the *Amy* structural gene are known in *Drosophila* belonging to more or less related taxonomic entities (see for example Brown *et al.*, 1990, for duplication in *D. pseudoobscura*), raising the question of the ancientness of this event. One can expect from the comparison of the gene arrangements and their structure at the molecular level to get a better understanding of the evolutionary history of this gene family.

We are pleased to thank Dr W. W. Doane and Dr A. J. Klarenberg for their helpful comments on this manuscript, and Dr Y. N. Tobari for supplying a photomap of the salivary gland chromosomes.

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