



The biochemical basis of insecticide resistance and determination of esterase enzyme patterns by using PAGE in field collected populations of *Drosophila melanogaster* from Muğla province of Turkey

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Abstract

Biochemical and molecular biology methods provide more specific information about the resistance potential or the development of resistance in field populations of living organisms. In this research, the enzymatic activities of Glutathione-S-transferase (GST), percent remaining acetylcholinesterase (AChE) and general esterase were measured in *Drosophila melanogaster* populations collected from different towns of Muğla Province. Furthermore, esterase enzyme patterns of these populations were determined by using polyacrylamide gel electrophoresis (PAGE). Percent remaining activities of AChE were found to be significantly higher in all strains compared to the activities of the susceptible one. Twenty one different esterase bands were detected when α - and β -naphthyl acetate were used as substrates and the bands were classified as 18 α -esterases, 2 β -esterases and 1 α/β esterase.

Key Words: Esterases, glutathion-s-transferases, acetylcholinesterases, *Drosophila melanogaster*, PAGE

Muğla ve ilçelerine ait *Drosophila melanogaster* populasyonlarında insektisit direncinin biyokimyasal temellerinin ve esteraz enzim profilinin PAGE yöntemi kullanılarak belirlenmesi

Özet

Biyokimyasal ve moleküler biyoloji teknikleri, doğal populasyonlarda, potansiyel veya gelişmekte olan direnç mekanizmaları hakkında çok spesifik bilgiler sağlamaktadır. Bu araştırmada, Muğla ve ilçelerinden toplanan *Drosophila melanogaster* populasyonlarında glutatyon-S-transferaz (GST), yüzde kalan asetilkolin esteraz (AChE) ve genel esteraz aktiviteleri ölçülmüştür. Bununla birlikte poliakrilamid jel elektroforezi (PAGE) yöntemi kullanılarak örneklerdeki esteraz enzim profilleri belirlenmiştir. Yüzde kalan asetilkolin esteraz enzim aktivitesi incelenen örneklerde duyarlı soya göre yüksek bulunmuştur. α ve β -naftil asetatın substrat olarak kullanılması ile toplam 21 esteraz bandı elde edilmiştir, bu bantların 18 tanesi α esteraz, iki tanesi β esteraz ve bir tanesi ise α/β esteraz olarak sınıflandırılmıştır.

Anahtar Sözcükler: Esteraz, glutatyon-S-transferaz, asetilkolinesteraz, *Drosophila melanogaster*, PAGE

Introduction

Insecticide resistance has been developed within natural populations of *Drosophila melanogaster*, although these insects are not usually direct targets of toxin applications. There are many advantages of using this insect for the study of insecticide resistance as a model organism, because for example, it has rapid life cycle, small number of chromosomes, ease of chromosome manipulation, and availability of its sequenced genome (Wilson, 2001; Miyo et al., 2001).

Esterase enzymes play an important role in conferring or contributing to insecticide resistance in insects. This has been shown in aphid *Myzus persicae* (Field and Devonshire, 1998), mosquitoes, *Culex quinquefasciatus* and *C. pipiens* (Guillemaud et al., 1997), blowfly, *Lucilia cuprina* (Campbell, 1998) and housefly *Musca domestica* (Claudianos et al., 1999; Taskin and Kence, 2004). The biochemical and physiological properties of esterases were previously studied (Healy et al., 1991; Oakeshott et al., 1993). In insects, the esterase enzyme patterns have shown high rates of intraspecific and interspecific variations (Nascimento and Campos Buicudo, 2002). Possibly, gene duplication followed by divergence of duplicated genes from the ancestral gene is the origin of at least part of this variability (Oakeshott et al., 1993; Nascimento and Campos Bicudo, 2002).

Acetylcholinesterase enzyme (AChE), a key enzyme in insect central nervous system, terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine. Resistance associated with modification of AChE makes it less sensitive to inhibition by organophosphates (OP) and carbamate insecticides (Feyereisen, 1995). Several point mutations have been identified in *D. melanogaster* (Mutero et al., 1994; Menozzi et al., 2004) and *M. domestica* Ace genes, encoding AChE (Kozaki et al., 2001). It was reported by Charpentier and Fournier (2001) that there is a significant correlation between the AChE amount and resistance to parathion, an OP, in the field collected populations *D. melanogaster*.

Glutathione-S-transferase enzymes (GST) play an important role in detoxification of xenobiotic compounds including insecticides. GSTs can produce resistance to a range of insecticides by conjugating reduced glutathion (GSH) to the insecticide or by its primary toxic metabolic products (Hemingway et al., 2000; Enayati et al., 2005). In the insecticide resistant

strains of housefly, the OP resistance is associated with elevated levels of GST activity compared with that of susceptible flies (Zhou and Syvanen, 1997). Enhanced GST activity, together with enhanced P450 activity, was mentioned in resistant *D. melanogaster* strains (Staal, 1975) (literature cited in Wilson, 2001).

To our knowledge, there have been no literatures published on the mechanisms of insecticide resistance in Turkish *D. melanogaster* populations. Therefore, the first purpose of our study was to understand the molecular nature of OP insecticide resistance in *D. melanogaster* populations collected from Muğla Province. The second aim was to determine the esterase enzyme patterns, which plays an important role in resistance, by using polyacrylamide gel electrophoresis (PAGE) in these populations.

Materials and methods

Drosophila melanogaster populations

D. melanogaster samples were collected from farms and garbage disposal sites at 12 different towns of Muğla Province in the summer of 2005. The towns are; Fethiye, Ortaca, Dalaman, Köyceğiz (Gökova), Marmaris, Datça, Muğla, Yatağan, Milas, Kavaklıdere, Bodrum, and Ula. Additionally, a *D. simulans* population collected from Ula was used for comparative purposes in all biochemical tests. The Kavaklıdere strain was used only for PAGE analysis because there was no sufficient number of flies of this strain. *D. melanogaster* and *D. simulans* samples were distinguished from each other based on the observation of male's genitalia as a key character under a light microscope. We have collected 50-70 individuals from each location and cultured in the laboratory to increase the numbers. Strains have been maintained in bottles containing corn starch, yeast and agar medium at the temperature of 25°C ± 1 °C. The susceptible strain, which had been raised in our laboratory and has never been exposed to insecticides for many years, was used for comparative purposes. The LD50 value for OP of this strain was much higher than that of other populations.

Bioassay

According to the information obtained from the Directory of Health in Muğla, an OP insecticide,

chlorpyrifos, was used to control pest species in 2005, in Muğla region from where our strains were collected (personal communication). However, our communication with the local community has revealed that the most preferred insecticide was malathion, due to its low cost and widespread use for numerous years. Therefore, we have chosen malathion in our experiments, which was 98.1 % technical grade. The filter-paper-contact method, as applied in Miyo et al. (2000), was adopted for the bioassays. In the same study it was reported that the LC₅₀ value for *D. melanogaster* was 2.5 µg/cm². So, in our experiments, we used six different concentrations ranging from 0 to 5 µg/cm² and reached the LD₅₀ value for our strains was 1.416 µg/cm². This concentration was used to determine OP resistance status of field collected populations. Each test was run twice and every replicate contained thirty flies. Additionally, a control treatment containing only the same amount of acetone but no malathion was run with each test simultaneously to observe the effect of diluent's on survival. Another control with no treatment was also used. For both controls no mortality was observed.

Preparation of extracts for enzyme assays

For enzyme activity assays, the extracts were prepared from, four-five days old, twenty individual flies that were sampled from each population of twelve different towns of Muğla province. In AChE and general esterase activity assays, the flies were homogenized in a phosphate buffer (containing %1 triton X-100) and for determination of both enzyme activities, the extracts from the same twenty individuals were assayed. However, in the case of detecting GST activities, different twenty individual flies were used due to the difference in the homogenization buffer (Tris-HCl).

Acetylcholinesterase (AChE) assay

AChE activities were measured according to the method of Moores et al. (1988). The procedure was done as follows; twenty individual flies were homogenized in 0.1M phosphate buffer, pH=7.5 (containing %1 triton X-100). AChE activity was measured by using acetylthiocholine iodide as substrate. The released thiol product was detected colorimetrically upon its reaction with DTNB (5,5'-

dithiobis-2-nitrobenzoic acid). Activities were measured at 405 nm, using Labomed UVD2950 spectrophotometer, with or without an inhibitor, maloxon. Percent remaining activities were calculated as: Mean inhibited activityX100/uninhibited activity.

Glutathione-s-transferase (GST) assay

GST activities were measured according to Habig et al. (1974) with some modifications. In this method briefly, twenty individual flies were homogenized in 300µl of 225mM Tris-HCl buffer (pH 7.5). Then, the enzyme activities were measured by using CDNB (1-chloro-2-4-dinitrobenzene) as a substrate as nmol/min/mg at 344 nm with a spectrophotometer (Labomed UVD2950).

General esterase activity assay

General esterase activities were determined based on the method of Zhu and He (2000). Twenty individual flies used were homogenized in 0.1M phosphate buffer, pH=7.5 (containing %1 triton X-100). α- and β- naphthyl acetates (α- and β-NAs) were used as substrates. Reaction was stopped by adding fast blue B-SDS solution and absorbance was determined at 600 nm for α-NA and at 560 nm for β-NA.

Protein assay

The protocol from Bradford (1976) was followed. *Native polyacrylamide gel electrophoresis (PAGE)* Esterase patterns of the flies were determined in 8% polyacrylamide gels (Souza-Polezzi and Bicudo, 2005). For better visualization of bands in the gels, 4 flies, at 8-10 days-old, from each strain were homogenized in 50 µl of the homogenization solution, 1.5 M Tris-HCl, pH 8.8, plus 10% glycerol. The supernatant (25 µl) was then subjected to electrophoresis for 5 hr at 4°C, using constant 30 mAmp. Esterases were identified as bands developed by the catalysis of α- and β-NA's as substrates. Bands showed the following characteristic colors: black (for those that hydrolyze α-NA), red (for those that hydrolyze β-NA) and magenta (for those that hydrolyze both α and β-NA's as substrates). Based on these results, they were named as α-esterases, β-esterases, and α-β esterases, respectively.

Table 1. Mean percent (N=2) LD₅₀ values of OPexposed *D. melanogaster* populations from Muğla Province.

Control	Bodrum	Dalaman	Datça	Fethiye	Gökova	Marmaris	Milas	Muğla	Ortaca
63.3±4.7	30±4.7	23.3±4.7	35±7.1	41.7±7.1	28.3±2.4	25±7.1	35±2.4	31.7±2.2	25±2.4

Data analysis

Data were analyzed using the SAS version 8.0 software (SAS Institute Inc., Cary North Carolina, USA). Bartlett's test (Zar, 1999) performed prior to ANOVA indicated that variances of a given enzyme activity among sampling locations were not homogeneous. Hence, non-parametric Kruskal-Wallis test were used for the comparison of enzyme activities (N=3) among sampling locations. Data from OP bioassay were first arc-sine square root transformed, then also analyzed with one-way ANOVA. When ANOVA detected significant differences ($p < 0.05$) among mean percent survivals or mean enzyme activities, Tukey's studentized range procedure (HSD) was used to separate the means into groups.

Results

Bioassay data

The results of OP bioassay are shown in Table 1. A broad range of susceptibility to malathion was observed in the populations tested. The populations in Yatağan and Dalaman, which are among the most industrialized areas in Muğla, were found to be significantly resistant to malathion insecticide when compared with the control strain, which was the most sensitive strain. There were no differences between the populations of Marmaris, Ortaca, Gökova, Ula, Muğla, Datça and Milas populations in terms of the resistance.

The mean percent remaining AChE activities, based on the diagnostic concentration of malaoxon, of the strains are given in Figure 1. Remaining mean percent AChE activities of the strains could be categorized in four groups; high, moderately high, moderate and low. Compared to control, all populations showed 1.2 to 2.2 fold higher insensitivity to malaoxon, suggesting that the target site for AChE insensitivity is likely to be the main OP insecticide resistance mechanism in the strains.

GST activity

CDNB-GST activities in field populations varied from 7.2 ± 1.2 to 13.8 ± 2.1 nmol/metabolized CDNB min/mg (Figure 2). Statistical analysis indicated that, populations were grouped into five different classes based on their GST activities; highest, moderately high, high, moderate and low. The populations collected from Ortaca, Muğla, Yatağan, and Datça had significantly lower GST activities than the control. However, the Marmaris population was not significantly different from the control. The other strains showed statistically higher activities for GST substrates than the control one. It seems that *D. melanogaster* field populations from Muğla province are composed of mixture of different GST alleles with different detoxification capacities.

General (total) esterase activity

In all strains, the α -NA substrate preference over β -NA was observed. This result agrees well with the results obtained from using non-denaturing PAGE analysis (α -esterase bands were observed thicker and stained more intensively). The substrate preference of α -NA over β -NA might be related with their feeding pattern of the insects in their natural environment. The results of esterase activity obtained by using α and β -NAs as substrates are given in (Figure 3 and Figure 4, respectively). Among all the tested strains, the Dalaman strain had the highest α -NA hydrolyzing activity compared to the activities of all other strains. For the substrate of β -NA the Ortaca strain showed the highest activity of $44.8 \mu\text{mol}$ produced β -naphthol/min/mg protein.

Esterase banding patterns

The esterase band patterns of the populations and the frequencies of each esterase bands detected in the native PAGE are presented in Figure 5. In all populations, a total of 21 esterase bands were detected

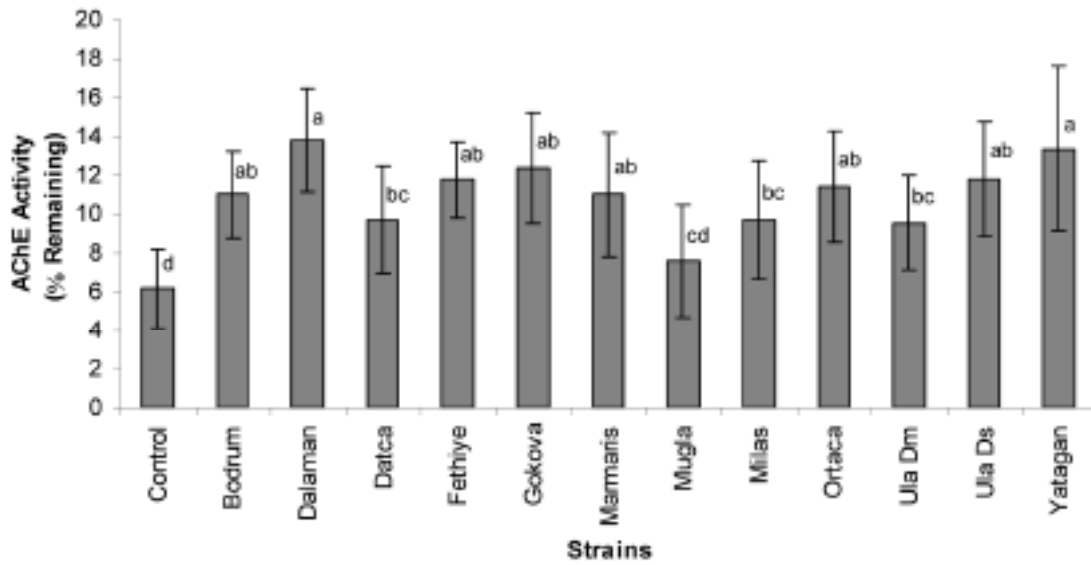


Figure 1. Mean (\pm SD, N=3) mean percent remaining acetylcholinesterase enzyme activities of *Drosophila melanogaster* populations. Means followed by the same lowercase letter are not significantly ($p>0.05$) different.

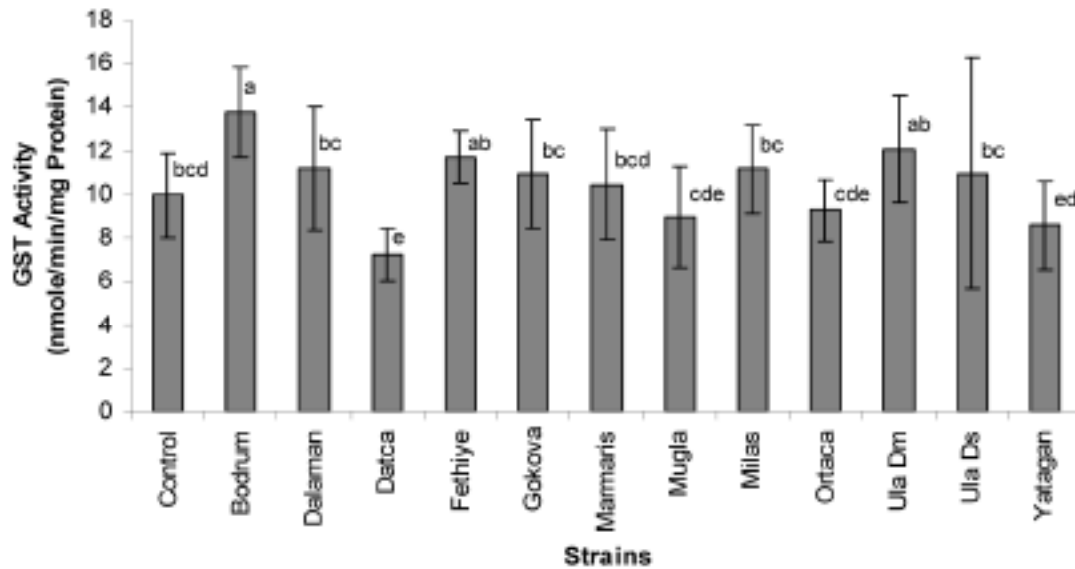


Figure 2. Mean (\pm SD, N=3) Glutathione-S-transferase enzyme activities of *Drosophila melanogaster* populations. Means followed by the same lowercase letter are not significantly ($p>0.05$) different.

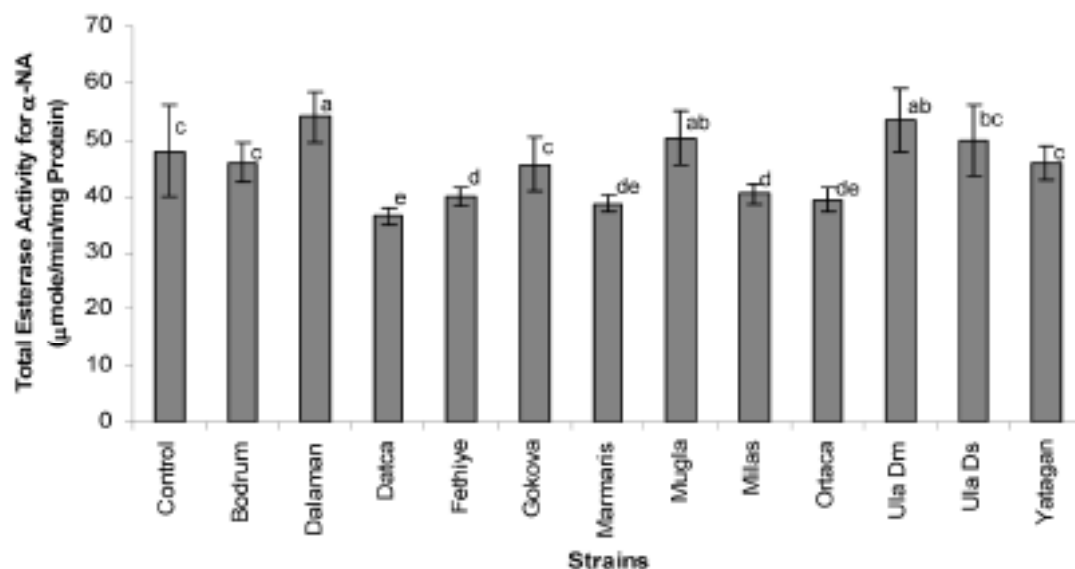


Figure 3. Mean (\pm SD, N=3) of total esterase enzyme activities (using α -naphthyl acetate as substrate) of *Drosophila melanogaster* populations. Means followed by the same lowercase letter are not significantly ($p>0.05$) different.

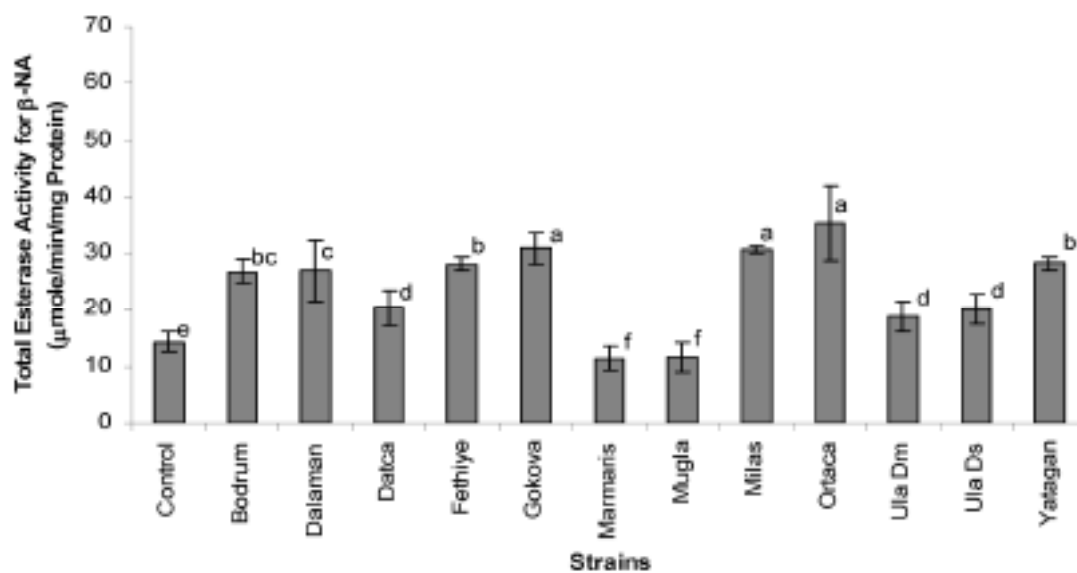


Figure 4. Mean (\pm SD, N=3) total esterase enzyme activities (using β -naphthyl acetate as substrate) of *Drosophila melanogaster* populations. Means followed by the same lowercase letter are not significantly ($p>0.05$) different.

and named according to their relative mobilities in the gel. Eighteen of them (EST-1 to EST-8, and EST-12 to EST-21) were black hydrolyzing the substrate α -NA and thus they were classified as α -esterases; two of them (EST-9 and EST-11) were red hydrolyzing the β -NA and were classified as β -esterases; and one of them (EST10) were magenta hydrolyzing both α - and β -NAs being classified as α/β -esterases. EST-8, EST-9, EST-13, EST-14 and EST-20 were common within and among populations nearly in all *D. melanogaster* populations suggesting that these proteins are essential for the organisms. Other bands were observed at different frequencies in all samples (Figure 5).

Discussion

There are several methods that have been developed for detecting resistance in insects. Usually, laboratory bioassays are performed on the progeny of field collected insects for detecting insecticide resistance. Traditional bioassay methods depend on measuring of LD50 or LC50 values, which are based on the criteria of insecticide amount required for killing 50% of a test population. In order to determine the resistance ratio of a field population, these values are divided by the LD50 of a susceptible laboratory population. Although these bioassays are easy to perform, they have several disadvantages. First, they are time-consuming since they need many individuals for testing one insecticide. Second, and most importantly, they do not provide a quantitative measure of potential resistance in individual strains or clones. On the other hand, biochemical methods and molecular biological techniques overcome these disadvantages in many aspects by reducing the sample size and giving more specific information about the resistance potential or the development of resistance in field populations (Kristensen, 2005; Han et al., 1998). In this study, the biochemical basis of OP insecticide resistance and the esterase enzyme patterns were investigated in Turkish *D. melanogaster* populations for the first time in literature.

Point mutations that modify the target AChE have been identified in OP-resistant strains of the fruit fly *D. melanogaster* (Mutero et al., 1994). The modified AChE, comparing with the wild type, decreased the sensitivity to inhibition by OP insecticides by two to four folds (Chen et al., 2001; Shi et al., 2004; Menozzi et al., 2004). It was also reported by Charpentier and

Fournier (2001) that there is a positive correlation between AChE amount and the resistance to an insecticide parathion, an OP. In our experiments, the results for AChE were the most clear-cut. Consistent with the bioassay results, the populations of Dalaman and Yatağan are the most OP insensitive considering the percent remaining AChE activities. Except for the population of Muğla, the percent remaining AChE activities in the rest of the populations are not significantly different from each other, including *D. simulans* populations from Ula. The percent remaining AChE activities obtained from all the populations were significantly higher than that of the control strain. This result suggests that the insensitivity of AChE to OP could be the main resistance mechanism. As mentioned by Charpenier and Fournier (2001), this can be explained by the history of strains, flies from highly insecticide sprayed areas might gather resistance mechanisms such as combination of several mutations in Ace gene or overproduction of AChE. However, the strains from low amount of insecticide sprayed areas might be poor in resistance mechanisms, considering AChE.

Enhanced GST activity, together with enhanced P450 activity, was reported for resistant *D. melanogaster* strains (Staal, 1975) (literature cited in Wilson, 2001). In our experiments, for some strains, we did not observe significant differences in GST activities compared with the control. Probably, P450 enzymes play a more important role in detoxification of OP insecticides in our *D. melanogaster* populations.

For general esterase activity assays, we did not observe consistent relationship with the esterase enzyme activity (by using α - and β -NA's as substrates), some strains having lower activities than the control. The most probable reason for this, as explained in Ahmed and Wilkins (2002), when an insecticide enters an organism, before reaching its target site, it could meet with different enzyme and protein obstacles, as a result of interactions with these enzymes the insecticide is degraded. Although the activity level of esterase enzyme was not elevated in our experiments, their possible role of in resistance can not be underestimated.

The esterase patterns and phylogenetic relationships of *Drosophila* species in the saltans subgroup were previously reported by Nascimento and Bicudo (2002). In our experiments, EST-8, EST-9, EST-13, EST-14 and EST-20 which showed the

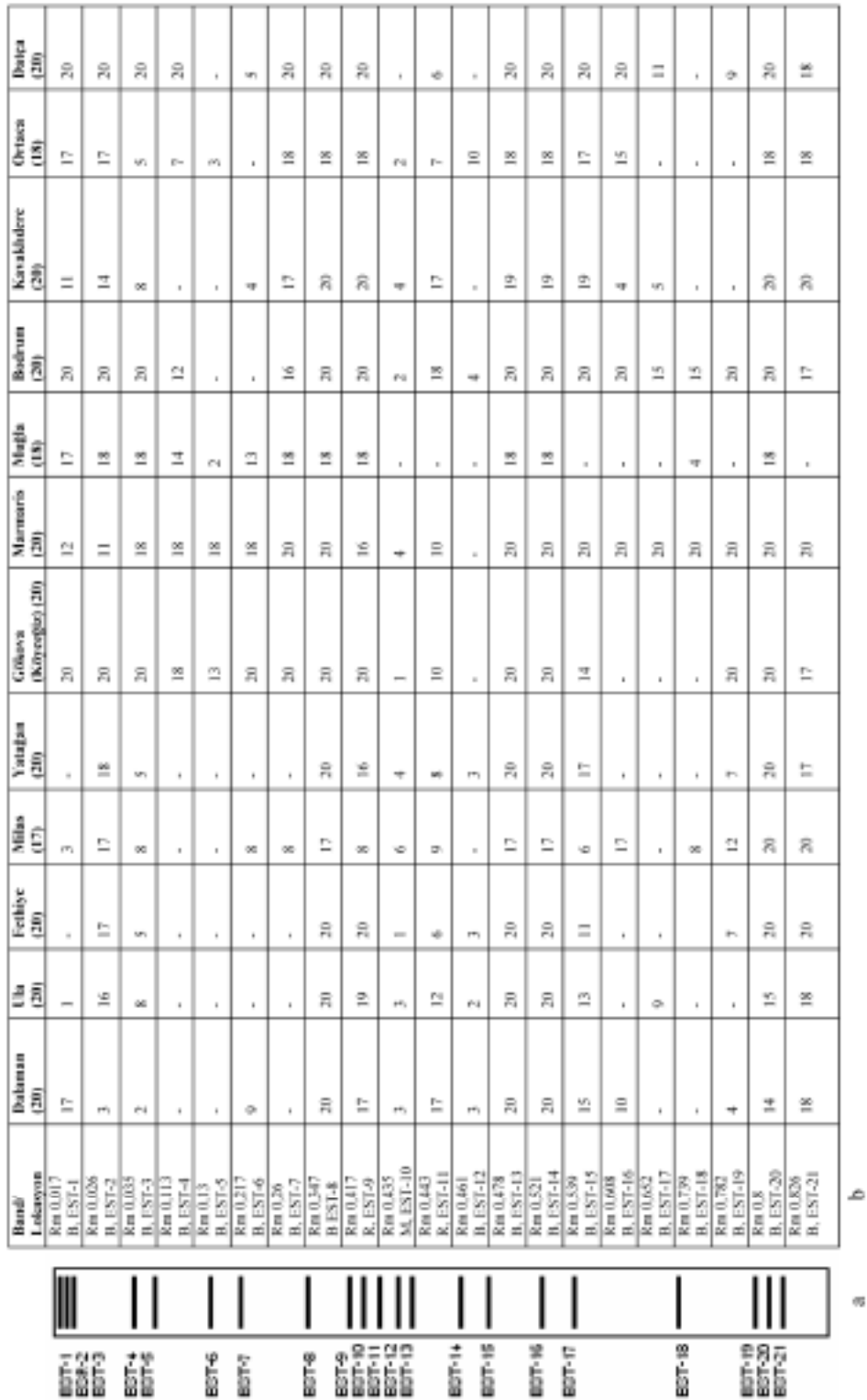


Figure 5. a) Esterase patterns of field collected populations of *Drosophila melanogaster* from Muğla Province of Turkey b) The number of esterase bands for each strain. R= red; B=black; M= magenta; Rm= Relative mobility. The number of lanes analysed from each population is shown in paranthesis.

greatest activity degree as indicated by intensity and staining were present in nearly all the samples analyzed. These enzymes are probably very important for the organism. The other bands were observed at different frequencies in all the samples. As also mentioned in Souza- Polezzi and Bicudo (2005), the different frequencies of esterase bands detected within and among populations in this study can be explained by the substitution of alleles under the environmental pressure or under the effect of genetic drift. The intensive use of insecticides and different pest management strategies can be considered as an important environmental pressure factors. Another reason for the frequency variations could be attributed to the age differences between the fly samples used. It was also stated by Menozzi et al. (2004) that “field populations are composed of mixture of different alleles with different sensitivities to each insecticide so the treatment with one pesticide would eliminate one allele but would select another one”.

This study demonstrated that there is a potential for AChE based OP resistance, in the collected populations of *D. melanogaster* in Muğla province. However, we did not observe a consistent relation with GST and general esterase activities and OP resistance on these populations. Biochemical methods are useful for detecting resistance mechanisms at the molecular level but unfortunately they don't enable us to identify the resistance mutations at DNA level. In order to determine the new pest management strategies the resistance status of field collected species should be tested at DNA level.

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