ESTERASE ISOZYME VARIATION AND POLYTENE CHROMOSOME ANALYSIS IN THE MELON FLY, *BACTROCERA CUCURBITAE* (DIPTERA: TEPHRITIDAE)

A Dissertation Submitted for the Fulfilment of the Requirements for the Degree of Master of Philosophy (M. Phil.) in Zoology (Entomology)

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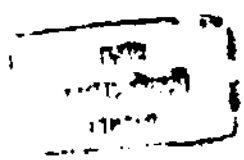

Dedicated

To

My Parents

DECLARATION

I do hereby declare that the work presented in this thesis entitled "Esterase isozyme variation and polytene chromosome analysis in the melon fly, *Bactrocera cucurbitae* (Diptera: Tephritidae)" was carried out by me under the joint supervision of Dr. Md. Fazlur Rahman, Professor, Department of Zoology, University of Dhaka and Dr. Reza Md. Shahjahan, Principal Scientific Officer, Institute of Food and Radiation Biology (IFRB), Atomic Energy Research Establishment (AERE), GPO Box No.- 3787, Dhaka- 1000, Bangladesh. The thesis or part of thereof has not been submitted in any form to any other university for any other degree. Moreover, no portion of this thesis has not been published earlier or sent anywhere for publication.

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CERTIFICATE

This is to certify that the thesis entitled "Esterase isozyme variation and polytene chromosome analysis in the melon fly, *Bactrocera cucurbitae* (Diptera: Tephritidae)" is a record of original research work carried out at the Institute of Food and Radiation Biology (IFRB), Atomic Energy Research Establishment (AERE), GPO Box- 3787, Dhaka- 1000, Bangladesh by Mr. Md. Hasanuzzaman, M. Phil. Regn. No.- 213, Session- 1998-99, under our joint supervision. We further testify that all data presented in this thesis are based on his own observations and no portion thereof has been used in any other thesis for a diploma or degree. The necessary help rendered by any person during the course of investigations and the sources of literature have been duly acknowledged.

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ABSTRACT

The melon fly *Bactrocera cucurbitae* (Coquillett) is well known as a destructive pest of fleshy fruits and vegetables in many parts of the world including Bangladesh. Esterase (EST) isozyme banding patterns were observed on 5% Polyacrylamide Gel Electrophoresis (PAGE) during the different life stages (i.e. egg, larva, pupa and adult) and also sweet gourd, which was used as larval food. Two bands, EST-1 and EST-2, showing seven allelomorphs were found. There were four electromorphs in EST-1 (Est-$1^{0.46}$, Est-$1^{0.58}$, Est-$1^{0.87}$ and Est-$1^{1.00}$), had a high mobility and close to the anode and EST-2 had three allelomorphs (Est-$2^{0.17}$, Est-$2^{0.27}$ and Est-$2^{0.37}$), showed a low mobility and close to the cathode.

The standard photographic maps of salivary glands polytene chromosomes were also observed in the different larval stages (1^{st} and 2^{nd} instar larvae) of *B. cucurbitae*. In second instar larvae, the whole polytene genome had been mapped by dividing it into 100 sections and the sub sections were lettered. The banding pattern was moderately developed here. The left end of the chromosome is characterized by dark bands in 4A, 6 and 14A. Sections 9 and 14B, each bears one pair faint bands. On the other hand, 15 comprises one pair moderately dark bands. The expanded regions in sections 3, 16 and 20 are not typical puff, rather section 16 bears a dotted band. The tip (section 1) has faintly stained two bands. Weak points are

found in 2 and 19. Deeply stained bands occur in sections 30, 31 and 41. Sections 33 and 40 are characterized by dotted bands; 39 and 42 comprise two moderately developed puff regions. Breakage occurs at 26. Sections 52, 55- 57 are characterized by a series of dark bands. Sections 48 and 50 have similar single band. There is a poorly developed puff region in section 58. Lightly stained two pairs of bands present in section 68. Sections 72, 73, 74 and 79 possess single band. One pair lightly stained bands occurs at 77 and 78. There is a prominent puff in section 80 together with a dark band immediately proximal to it. Weak points are found in 63, 76 and 81. Section 83 possesses three clear bands. A series of lightly stained and deeply stained bands are found in the sections 87 and 95. Section 100 appears only as a well-developed puff in the total polytene chromosome complement. Three weak points are found in 91, 97 and 98. In the case of 1st instar larvae, salivary gland cells were at early stages of maturity. So the banding pattern of polytene genome was not developed at this stage.

This observation showed that *B. cucurbitae* is an excellent genetical material for esterase isozyme and polytene chromosome analysis. The investigation has been proved to be very useful for obtaining more detailed genetic information on the natural populations of this pest.

Chapter-1

Introduction

INTRODUCTION

Bactrocera cucurbitae (Coquillett), the melon fly is a diptera of the family Tephritidae, which worldwide consists of around 4,000 species of which 250 are of economic importance. The species belongs within the sub-family Dacinae and the tribe Dacini. It is difficult to say precisely when the species was introduced to the region. The first identifications were made in Mauritius in 1960 and in Reunion Island in 1972. The species has probably been introduced to the Mascarenes from the Indian sub-continent. The species has been found in Iran and is widely distributed across from Pakistan, India, Bangladesh to China, Taiwan, Indonesia and the Philippines. It is also found in New Guinea and neighbouring Pacific Islands and has been recorded in Guam, the Marianne Islands and Hawaii. It has been introduced to Africa (Egypt, Kenya, Tanzania), and of concern to the western Indian Ocean, to Mauritius and Reunion Island (**Plate 1**). Melon fly has been eradicated from California and from the Ryukyu Islands of Japan by means of the Sterile Insect Technique (Vayssieres and Coubes, 1999).

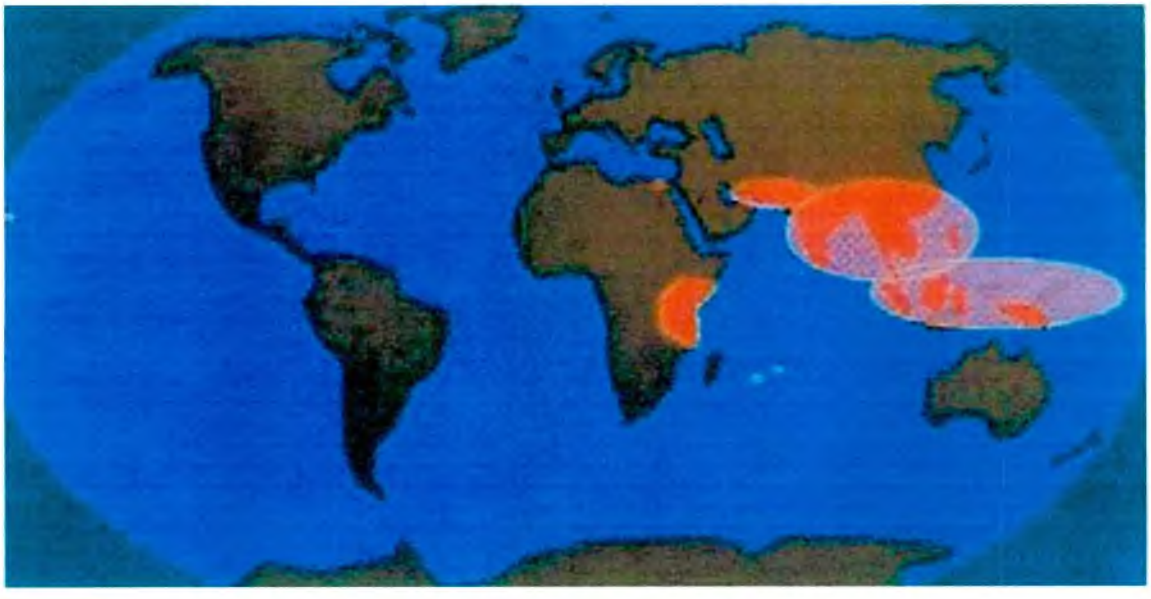


Plate. 1. World repartition of *Bactrocera cucurbitae* (**Source:** Centre de Cooperation Internationale en Recherche Agronomique pour le Developpement, CIRAD).

The species attacks principally cucurbit crops but it is polyphagous attacking altogether 16 plant species in Bangladesh (Kabir *et al.*, 1991). An understanding of the genetics of this species is essential for the development of novel control methods, particularly sterile insect release.

BACKGROUND OF ISOZYMES

Isozymes are multiple forms of a single enzyme, which often have different isoelectric points and therefore can be separated by electrophoresis (King, 1974). Hunter and Markert (1957) developed a technique, known as zymogram technique for determining enzyme heterogeneity. They combined the starch gel electrophoresis method of Smithies (1955) with histochemical staining. Finally, Markert and Moller (1959) were able to separate lactate dehydrogenase enzyme into five distinct forms, which they called isozyme. According to them, isozymes may be defined as multimolecular forms of enzymes, which are derived from the same organ or tissue and have at least one substrate as common. Later, the term isozyme was first used by Wroblewski and Gregory (1961) and it has been recommended by the Standing Committee on enzymes. The term has been defined by different investigators in different ways but the original definition of Markert and Moller (1959) has been widely accepted.

Many enzyme loci separated for several electrophoretically detectable variants in natural populations. Population biologists have used the electrophoretic technique to answer questions of paramount importance in theoretical and applied genetics (Nevo *et al.*, 1984), associated with pest management.

Loukas, M. (1989) reported that isozyme analysis has been used: (1) To estimate the genetic distances between different populations. (2) To study the processes accounting for the maintenance of genetic variability in natural populations and the role of this variability in this populations and the role of this variability to species adaptation. (3) To monitor the genetic structure of natural and artificially reared populations. (4) To study the levels of genetic variation and the distribution of allele frequencies in natural populations. (5) To trace the introduction and subsequent dispersal of each species.

Many other researchers also used to the isozyme variability: (1) To establish linkage groups (Malacrida *et al.*, 1988) for genetic manipulations (Saul, 1986). (2) To study the sperm priority and multiple mating (Zouros and Krimbas, 1970). (3) To study the creation of host races that may occur by the adaptation of species to new plants (Bush, 1969). (4) To study the effects of insecticides on

polymorphisms (Tsakas and Krimbas, 1970). (5) To study the development differences and tissue specificity (Malacrida *et al.*, 1983). (6) To develop a genetic sexing system (Riva and Robinson, 1986 and Robinson *et al.*, 1986). (7) To identify the insect species (Wool *et al.*, 1989 and Khuda-Bukhsh Sen, 1990). (8) To study the insecticides resistance mechanisms in insect (Gorphade & Wilkins, 1987; Chen & Sun, 1988; Baan *et al.*, 1990; Saito, 1990; Sloderbeck *et al.*, 1991; Abdel *et al.*, 1992; Field & Devonshire, 1992; Ono, *et al.*, 1994; Bloch & Wool, 1994; Sakata & Miyata, 1994; Moores *et al.*, 1996 and Grafton-Cardwell *et al.*, 1998). (9) To identify the biotype of insects (Abid *et al.*, 1989; Liu *et al.*, 1992 and Costa *et al.*, 1993). (10) To identify the insect subspecies (Gruppe, 1988).

BACKGROUND OF CHROMOSOMES

For the period of sixty years, *Drosophila melanogaster* has remained the favourite insect for genetical research in multicellular organisms (Pain and Smith, 1973). The economic impact of the melon fly, *Bactrocera cucurbitae* has resulted in considerable efforts to devise control and eradication methods, particularly Sterile Insect Technique (SIT).

The means of control of the fly are mainly based on chemical insecticides, although there is increasing interest in autocidal and

other genetic control techniques (Robinson, 1983). However, there is considerable interest in biological control, such as the sterile insect technique, pheromone traps and endotoxins (Karamanlidou *et al.*, 1991). Unfortunately, such methods, specially the SIT programme, are hampered by the absence of basic genetic and cytogenetic data on this species. Workable polytene chromosomes are especially favourable for autosomal studies allowing accurate analysis of euchromatic regions (Bedo, 1986).

The polytene chromosomes were first described by Balbiani in larval glandular tissues of dipterous insects in 1981. The unusual size of the salivary gland polytene chromosomes is a product of the type of growth. Salivary and other glands grow by enlargement rather than by duplication of individual cells which can be demonstrated by cell counts and measurements taken at different developmental stages of a larva. As a larva develops, the chromosome threads (chromonemata) in the cells duplicate themselves repeatedly, producing bundles. Many chromonemata are originated by duplication of each chromosome in the original cells and these are involved in the formation of the gland. Thus chromonemata are present as single giant chromosomes. Giant chromosomes are thus cablelike structures with many stands and are called polytene. Bands and interbands running crosswise on the giant chromosomes represent an

accumulation, through continuous duplication, of basophilic stainable regions. These regions are known as chromomeres. Giant polytene chromosomes thus correspond in linear structure with other individuals of the same species. The difference is that the duplicate stands (chromatids) are held together in bundles. They do not separate out to new cells through cell division. Polytene chromosomes are basically similar with other chromosomes. They contain DNA and protein in each strand. The continuous state of somatic synapsis makes the giant salivary chromosomes valuable for study. If one member of a homologous pair is altered by deficiency, duplication, inversion, or translocation, an irregularity occurs in pairing. Different kinds of chromosome modifications and their location on the chromosome can recognize by observing their characteristic irregularities. Terminal deficiencies in *Drosophila*, as reported by Lefevre (1976), could be distinguished by banding of an unpaired end of a salivary chromosome. About 5000 single cross bands have been noted on the four pairs of salivary chromosomes in *D. melanogaster*. This number was considered to be a minium approximation of the number of genes in that insect. Genes have been associated with individual bands.

The main uses of polytene chromosomes are: (i) to locate genes and (ii) to identify structural changes in chromosomes (Gardner & Snustad, 1975).

Any chromosome nomenclature system relies on accurate identification of each karyotype element in the genome. This is not a problem in polytene cells where unique banding patterns readily identify the chromosomes, the recognition of mitotic chromosomes can be more difficult because of the small number of morphological markers that are available. In many cases only gross characters such as relative chromosome length, arm ratios, and the presence of secondary constrictions can be used. If some of the mitotic chromosomes can not be distinguished in these terms, then problems eventually arise in establishing homologies between the mitotic and polytene complements.

The polytene tissues studied in *C. capitata* orbital bristle trichogen cells (Bedo, 1986 & 1987) and salivary glands (Zacharopoulou, 1987 & 1990) show only five polytene elements, corresponding to the five autosomes.

AIMS AND OBJECTIVES

The aims and objectives of the present study are as follows:

- To study the esterase isozyme patterns at the different life stages of the melon fly, *B. cucurbitae*.
- To study the banding patterns of salivary gland polytene chromosomes (1^{st} and 2^{nd} instar larvae) and construct a photographic map.
- To develop biochemical and cytogenetic markers through characterization of the isozyme profile and karyotype variants that would be helpful in devising pest management strategy by genetic manipulation.

Chapter-2

Review of Literature

REVIEW OF LITERATURE

Without literature review, it is very difficult to appraise the development of any field of study. In this brief outline of literature one may be able to see some relation between one research work and another so that the present research works do not appear completely isolated. In a more specific way efforts have been made to incorporate the latest developments in the genetical research field which facilitated my research works.

ISOZYMES

Burns and Johnson (1967) studied starch-gel electrophoresis of esterases of wild and laboratory reared individuals of *Colias eurytheme*. Their study indicated that populations in central Texas were exceedingly variable, consisting entirely of heterozygotes formed a large number of alleles at an autosomal locus controlling the production of an esterase designated EST E. Few wild individuals were generally identical at this locus. The commonest member of the allelic series is an apparent null allele that results no EST E activity.

Zouros *et al.* (1968) found the main bulk of adult esterases in the olive fruit fly *Dacus oleae* (Gmel) is controlled by two autosomal and independent genes, gene A and gene B. Gene A Synthesizes an

acetylcholinesterase and gene B a pseudocholine esterase or a lipase. Both genes are highly polylmorphic in natural population. More than 15 active alleles plus one silent allele have been found for gene A and more than 10 active alleles plus one silent allele in gene B.

Trebatoski and Craig (1969) studying the genetics of an esterase in *Aedes aegypti*, obtained results indicating that one esterase, existing in two forms distinguishable by electrophoresis in starch gels, was controlled by a pair of alleles at a single gene locus.

Townson (1969) found the esterase isozymes of individual *Aedes aegypti* by using the use of electrophoresis in polyacrylamide gels to separate esterases. Mosquitoes were found which differed in the mobility of the most prominent esterase band. Six different esterase bands have been detected, although all six are rarely present in one mosquito. All bands stained the same red colour but some stained more intensely than others. The most mobile esterase shows variability in both male and female. Female mosquitoes were screened in which the R_S of this component was 0.96. One variant had this esterase with an R_S of 0.92 and other had both 0.96 and 0.92 bands. The 0.96 band was typically the most heavily stained, where as the 0.92 component was generally weaker. One variant was obtained in which neither of these components was detectable but the

band of R_S 0.65 was intensified, indicating that the absence of 0.92 and 0.96 components was not due to the over-all low concentration of esterases.

Tsakas and Krimbas (1970) studied relation between adult esterase genotypes and survival to organophosphate insecticides in *Dacus oleae*. Dimethoate, an organophosphate insecticide, preferentially killed adult insects homozygous or heterozygous for the silent allele of esterase gene A. Thus dimethoate selected against the silent allele of gene A.

Krimbas and Tsakas (1971) found in laboratory experiments that the organophosphate insecticide dimethoate acted as a selective agent on gene A esterase polymorphism of adult *Dacus oleae*, selecting against the silent isoallele. However, in a small natural population, dimethoate did not seem *prima vista* to act as a selective agent; instead, observed gene frequency changes were a consequence of population size reduction (genetic drift).

Townson (1971) separated the esterases of individual *Aedes aegypti* by electrophoresis on polyacrylamide gel. Mosquitoes were found which differed in the mobility of the most intensely staining band. The following four phenotypes were seen: F (fast), which had

the more mobile band; S (slow), which had the less mobile band; FS, which had both fast and slow bands, and a null phenotype which had neither, though other bands stained normally. On the basis of single pair matings, it is believed that the F and S forms of the enzyme are controlled by two alleles, designated Est-α^f, and Est-α^s, and that a third allele Est-α^x occurs at the same locus. The F phenotype would be produced by genotypes f/f and f/x; the S by s/s and s/x; the FS by f/s; and the null phenotype by x/x. With P-nitrophenylacetate used as substrate, the Km values of the two forms of the enzyme were found to differ.

Georghiou and Pasteur (1978) investigated the esterase patterns of insecticide-resistant and susceptible strains of *Culex pipiens fatigans* Wiedmann, *Culex pipiens pipiens* L., *C. tarsalis* Coquillett, and *Anopheles albimanus* Wiedmann by starch gel electrophoresis. At least one highly active esterase was present in every organophosphate-resistant strain of *Culex* spp. A highly active esterase B2, catalyzing the hydrolysis of β-naphthylacetate and being suppressible by DFF® (S, S, S-tributyl phosphorotrithioate) was most probably associated with organophosphorous multiresistance in *C. p. fatigans* from California. Esterase B2 had no equivalent in chloropyrifos-resistant *C. p. pipiens* from France.

Kreutzer (1979) studied esterase isozymes in the mosquito *Culex erraticus* by using cellulose acetate electrophoresis and reported that there were at least 5 separate loci for esterase (EST) activity. Locus EST-1 had a single band of activity which was not present in larvae. Locus EST-2 also had a single band which was present in larvae and pupae but was lost about 5 days after emergence. Locus EST-3 had three alleles, locus EST-4 two alleles, and locus EST-5 three alleles.

Georghiou *et al.* (1980) stated that a highly active esterase associated with resistance to temephos, chloropyrifos, parathion, methyl parathion, malathion, and fenthion in *Culex quinquefasciatus* Say from California was coded by a single gene with 2 alleles : EST-B^{A}, coding a strongly staining esterase; and EST-B^{0}, coding a weakly staining esterase. EST-B^{A} was found to be dominant over EST-B^{0}. EST-B gene was localized on the same chromosome as the gene coding resistance and there was a strong possibility that these two genes were identical since no recombinants were observed.

Matthews and Craig (1980) analyzed 18 enzyme loci by the process of electrophoresis in local populations of the tree-hole mosquito *Ae. triseriatus* from north-western Indiana and south-western Michigan and revealed that genetic variability in these

populations was quiet extensive. The average heterozygosity (H) was 21.5%, and the proportion of polymorphic loci based on Criteria I and II was 0.720 and 0.495, respectively. The average genetic identify (I) was 0.994. Comparisons between oak and beech populations showed no tendency toward differentiation between oak and beech ecotypes.

Munstermann (1980) compared enzyme variation in the 4 taxa of the *Aedes atropalpus* group. Average heterozygosity over the 17 loci examined was low (3-10%), reflecting the inbreeding effects of colonization. Genetic distance (Nei's) between *Aedes atropalpus* and *Ae. epactius* was 0.45 or greater with 5 loci discriminating between the species. To a lesser degree, the 3 strains of *Ae. epactius* from different parts of its range were also genetically distinguishable. Specimens from a new habitat (discarded tires) outside the range of both species were morphologically *Ae. atropalpus* but showed some behavioral characters of *Ae. epactius*. These specimens were positively identified as *Ae. atropalpus* based on discriminating enzyme phenotypes.

Curtis and Pasteur (1981) found resistance to chloropyrifos and other commonly used organophosphate larvicides in *Culex quinquefasciatus* Say from several urban areas in Tanzania. Lower levels of resistance were found in laboratory strains of the complex of

C. pipiens L. collected in Sri Lanka, Egypt, Liberia and Brazil. Crosses of the Tanzanian strains to a more susceptible strain gave results consistent with causation of the resistance by a single gene or closely linked group of genes. Electrophoresis of one of the Tanzanian stocks indicated two esterase bands of high activity.

According to Bitondi and Mestriner (1983), starch gel electrophoresis utilizing different types of substrates and inhibitors made it possible to detect several esterases in crude extracts of *Apis mellifera*. Their results suggested that there were six esterase isozymes (esterases 1- 6) that differed not only in electrophoretic mobility but also in substrate specificity and inhibition properties. Some of the esterase isozymes were controlled by more than one allele. The frequency of these genetic variants was analyzed in four populations of *Apis mellifera* from several localities. Esterases 1, 2 and 4 did not exhibit developmental changes, but the electrophoretic profile of esterases 3, 5 and 6 varies during ontogenetic development.

Pashley and Rai (1983) employed numerical taxonomic methods to determine congruence between morphological and allozymic measurements in the *Aedes scutellaris* group of mosquitoes. In addition, allozyme alone were used to examine

relationships between three species groups (*Ae. aegypti, Ae. w-albus*, and *Ae. scutellaris*) of *Aedes*. Adult morphological traits and allozymes fit well with geographic distributions and classical taxonomic assignments, whereas larval traits were less reliable. The allozymic relationships at the group and subgroup levels were consistent with previous systematic treatments, in that all species fell into independent and cohesive clusters. However, the *albopictus* subgroup within *scutellaris* showed a slightly greater affinity to *w-albus* that to its closer relatives in the *scutellaris* subgroup.

Villani *et al.* (1983) studied inheritance and activity of some esterases associated with organophosphate resistance in mosquitoes of the complex of *Culex pipiens* L. Eighteen strains of the mosquito from Africa, Asia and Europe were bioassayed for resistance to chloropyrifos and electrophoresed and stained for esterases. Susceptible strains showed only low activity esterase bands. The resistant strains of *Culex quinquefasciatus* Say from hot countries (Nigeria, Sri Lanka, Tanzania, Thailand) all showed the same two high intensity esterase bands (Rm 0.60+0.82). Different patterns of high esterase were found in resistant *C. pipiens* strains from cooler localities in Nairobi, Kenya (Rm 1.00), and Montepellier, France (Rm 0.50). The high intensity esterase bands were probably due to alleles of the loci Est-1, Est-2 and Est-3, separated by crossover distances of

approximately 2.4 and 5.5 units respectively. The investigation concluded that high intensity esterases were reliable indicators of organophosphate resistance in mosquitoes of the *C. pipiens* complex, although the possibility of other resistance mechanisms mean that the lack of abnormally active esterases did not necessarily indicate the absence of resistance.

Harrington *et al.* (1984) conducted a survey of genetic variation in urban populations of *Aedes aegypti:* using starch gel electrophoresis. Ten enzyme loci were examined, 4 of which were found to be polymorphic (Hk. Mdh, Pgm, Pgi). The remaining loci were monomorphic (Ald, Ao, Fum, α-Gpd, Me, 6-Pgd). The expected heterozygosity of the total population (H_T) was 9.7%. When the expected total heterozygosity was partitioned, intrapopulation variation accounted for 9.3%; interpopulation variation contributed the remaining 0.4%. The results suggest that the *Ae. aegypti* population in Houston area is not genetically subdivided.

Corsaro and Munstermann (1984) identified *Culex* samples up to species level by starch gel electrophoresis. The enzyme migration rates for 4 species of *Culex* (*Cx. pipiens*, *Cx. restuans, Cx. salinarius* and *Cx. territans*) were compared. Six of the 14 enzyme banding patterns separated *Cx. pipiens* and *Cx. restuans*, while 1. "malic"

enzyme uniquely characterized each of the 4 species. None of the species diagnosed by the enzyme esterase.

Hemingway and Georghiou (1984) measured baseline general esterase levels spheetrophotometrically with the substrates α-and β-napthyl acetate for several *Anopheles* and *Culex* species. All the insecticide susceptible colonies showed similar levels of general esterase activity, which were much lower than, and clearly distinguishable from, those of organophosphate resistant *Culex tarsalis* and *Cx. quinquefasciates*.

Hemingway *et al.* (1986) reported that, in Sri Lanka *Anopheles nigerrimus* was resistant to a range of organophosphate and carbamate insecticides at both the larval and adult stages. Biochemical studies indicated that an alteration in acetycholinesterase was the basis of resistance rather than increased metabolic breakdown of the insecticides. In contrast, *A. culicifacies* was resistant only to malathion and closely related compounds containing a carboxylate ester bond. They concluded that agricultural pesticides were the sole source of selection pressure for resistance in *A. nigerrimus*, while in *A. culicifacies* pressure arose predominantly from antimalarial spraying.

Hemingway and Smith (1986) developed a simple field method for direct detection of an altered acetylcholinesterase (AChE) type organophosphate and carbamate resistance mechanism in individual insects. In initial field trials of this test in Sri Lanka, *Anopheles nigerrimus* Giles and *Culex tritaeniorhynchus* Giles were shown to contain this type of resistance gene, while a further nine species of mosquito had no evidence of it. Frequencies of the altered AChE gene in *A. nigerrimus* were estimated to be higher than those of *C. tritaeniorhynchus*.

Mouches *et al.* (1986) cloned an esterase gene from the mosquito *Culex quinquefasciatus* in λg+11 phage. The esterase gene in the mosquito was responsible for resistance to a variety of organophosphorous insecticides. This gene was used to investigate the genetic mechanism of the high production of the esterase B1, it encoded in OP-resistant *Culex quinquefasciatus* Say (Tem-R strain) from California. Adults of the Tem-R strain were found to possess at least 250 times more copies of the gene than adults of susceptible strain (S-Lab).

Raymond *et al.* (1986) investigated resistance mechanisms of a strain (MSE) of *Culex pipiens* L. which was collected in southern France in 1979 and highly resistant to chloropyrifos. The resistance

characteristics were compared to various organophosphates and carbamates in the absence or presence of synergists and determining the biochemical characteristics of four enzymes (esterases, glutathion-S-transferases, mixed function oxidases, and acetylcholine esterase) compared with a susceptible strain and a chloropyrifos resistant strain (S54) collected in the same area in 1960 and 1974, respectively. Chloropyrifos resistance in S54 was due to a detoxifying esterase, whereas resistance in MSE was associated with an acetylcholinesterase insensitive to the inhibition by chlorpyrifoxon and some carbamates, and with an increase of oxidative metabolism.

Chang and Whalin (1987) examined using alpha-naphthyl acetate, trans-permethrin, cis-permethrin, malathion and fenvalerate as substrates. Eight esterases were resolved from body homogenates of the brown planthopper with pI values in the range of 4.3 to 5.3 and designated as E1- E8. All esterases hydrolyzed alpha-naphthyl acetate, trans-permethrin, cis-permethrin, malathion at different rates. Fenvalerate was the most stable substrate to hydrolysis and was hydrolyzed by E1 to E8 except E3.

Georghiou *et al.* (1987) cloned an esterase gene in Tgtll phage from the mosquito *Culex quinquefasciatus* that is responsible for resistance to a variety of organophosphorus insecticides. This gene

was used to investigate the genetic mechanism of the high production of the esterase B1; it encodes in Op-resistant *Culex quinquefasciatus* Say (Tem-R strains) from California. Adults of the Tem-R strain were found to possess at least 250 times more copies of the gene than adults of a susceptible strain (S-Lab).

Gorphade and Wilkins (1987) examined three strains of the brown planthopper and were compared in the laboratory. Total esterase activity of the male and female brachypterous adults measured using a colorimetric method, was appreciably higher in the Japanese 2 population. The esterases hydrolysing 1-naphthyl acetate were separated into six bands of polyacrylamide gel electrophoresis. The resistant Japanese 2 strain showed higher activity in the main E5 band than the susceptible Japanese 1 strain.

Hearth *et al.* (1987) studied malathion resistance in field populations of *Anopheles culicifacies* B in Sri Lanka. The frequency of individuals with high esterase activity did not correlate well with resistance in the two field populations. This suggested that a qualitative rather than a quantitative change in esterase activity might be involved in this resistance. It was predicted that the resistance in *A. culicifacies* would be malathion specific and inherited as a single semidominant characteristic.

Hemingway *et al.* (1987) reported that larval specific carbamate resistance in *Anopheles subpictus* Grassi in Sri Lanka was produced by agricultural selection pressure and appeared to be correlated with high esterase activity. High esterase activity was found in both larvae and adults, but one of the larval elevated bands was not present in the adult, and two other adult bands were not found in the larvae. Broad spectrum organophosphate resistance was found in both the larvae and the adults and was associated with an increase in mixed-function oxidase activity.

Chen and Sun (1988) found that toxicity of permethrin could be enhanced about 50 times by tributylphosphorotrithioate, on inhibitor of esterases, in the four field strains of smaller brown planthopper (SBPH). Resistance to permethrin, and very possibly to malathion, is closely related to hydrolytic degradation by esterases. This resistance mechanism resembles what was found earlier in the brown planthopper (BPH). In BPH, high esterase activity associated with organophosphorous and carbamate resistance may have conferred a major part of the resistance to permethrin and other primary alcohol ester pyrethriods.

Gruppe (1988) differentiated three subspecies of *Myzus cerasi* in Central Europe. Their esterases differed constantly from each other. This was true for 234 samples from different populations, e.g. 171 samples from *Prunus avium* and 63 from *P. cerasus*. However, *M. cerasi* str. is characterized by two esterases with R(f)-values of 0.9 and 0.4; whereas *M. cerasi*, *P. avium* by three esterases with R(f)-values of 0.9, 0.4 and 0.2.

Magnin *et al.* (1988) tested a strain of *Culex quinquefasciatus* for resistance to various insecticides. Low but consistent resistance ratios (RR=3-4) were observed for five organophosphates and two carbamates. Use of the synergist S, S, S-tributylphosphorotrithioate (DEF) showed that esterases or glutathione-S-transferases played a major role in organophosphate resistance. Two highly active esterases, A2 and B2, were detected in mosquitoes studied. Selection with chloropyrifos increased significantly the frequency of these enzymes, confirming that they were involved in organophosphate resistance. Results on deltamethrin resistance suggested that oxidases were responsible for a part of the resistance to deltamethrin and that some other mechanism, most likely knock-down resistance, was involved in resistance to this insecticide and to DDT.

Abid *et al.* (1989) reported that the seven isolates of greenbugs, *Schizaphis graminum* (Rondani), and one isolate each of the orn leaf aphid, *Rhopalosiphum maidis* (Fitch), and the yellow sugarcane aphid, *Sipha flava* (Forbes) were tested for diagnostic isozymes. Among 12 isozyme systems tested, six gave no reaction with aphid extracts. Anodal and cathodal esterase showed isozyme patterns for aphids and corn or sorghum extracts, but the banding patterns did not show any differences among aphid species.

Saito (1989) was collected 39 populations of *Aphis gossypii* from the field in Japan and tested for susceptibility to several insecticides and for esterase activity. Aphids with high esterase activity were observed frequently in organophosphate-resistant populations.

Wool *et al.* (1989) studied electrophoretically adults of some species of whiteflies in Israel and Colombia. The patterns of esterase and alpha-glycerophosphate dehydrogenase isozymes were species-specific and may be used to identify whitefly adults.

Baan *et al.* (1990) observed that esterase detoxification activity was higher in *C. pyricola* than in the mirid predator *Deraeocoris brevis*, whereas, glutathione S-transferase and

cytochrome P-450 monooxygenase activities were similar in predator and prey.

Khuda-Bukhsh Sen (1990) observed non-specific esterase profiles in *Aphis gossypii*, *A. craccivora* and *Lipaphis erysimi* by using polyacrylamide gel electrophoresis. Samples of *L. erysimi* produced 2 esterase bands, while those of the other species produced a single band.

Saito (1990) was determined individual esterase activity in 124 populations of *Aphis gossypii* from 16 plants in Japan. The populations from each plant had different frequency distributions. Aphids with high esterase activity were observed frequently on Cucurbitaceae but not on Solanaceae, while strawberries and chrysanthemums supported aphids with both high and low esterase activity. It is suggested that insecticide resistance in this aphid is closely associated with biotypes.

Raymond *et al.* (1991) stated that distribution of electrophoretically distinct variants of overproduced esterases A and B in *Culex pipiens* was geographically restricted, with the exception of esterases A2 and B2, always found together throughout at least three continents. To determine whether this situation was due to

migration or to a high mutation rate, esterase B structural genes and their flanking regions were compared by sequence and/or restriction fragment length polymorphism analysis. Whereas structural genes were similar, flanking regions of electrophoretically dissimilar esterases B varied considerably. In contrast, flanking sequences of esterases B2 from different geographical locations (Africa, Asia, North America) were identical. These results suggested that amplified esterase B2 genes originated from an initial event that subsequently spreaded organophosphate insecticide resistance by migration.

Sloderbeck *et al.* (1991) evaluated resistance to parathion and chlorpyrifos-methyl of 2 strains of *Schizaphis graminum* by using a vial-residue bioassay technique. The tests, repeated 3 times over a 3-month period, detected resistance to parathion and chlorpyrifos-methyl in an aphid colony from a parathion-failure sorghum field when compared to a 3-year-old, susceptible greenhouse colony. A gel electrophoresis test indicated that aphids from the parathion-failure field had an esterase band 6 times as dense as the band obtained from the susceptible colony.

Abdel *et al.* (1992) found that insecticide resistance in *Myzus nicotianae* from different localities of the Southeastern USA to exist mainly in red-bodied aphids. The frequency distribution of esterase

activity in individual *M. nicotianae* collected from a tobacco fields and greenhouse indicated that organophosphate (OP) resistance was always linked to high carboxylesterase activity towards 1-naphthyl acetate. Two approaches to speed up the detection of resistance genotypes is described. The first used malathion as a reporter molecule to rapidly distinguish between OP resistant and susceptible *M. nicotianae*. A time threshold of 50 min following exposure to 50 p.p.m. malathion in a water suspension accurately discriminated between susceptible and resistant phenotypes. In the second approach, assays of carboxylesterases from *M. nicotianae* using the acetates and propionates of 1- and 2-naphthol, and 4-nitrophenol, were performed to optimize and better understand the esterase-discriminating activity between resistant and susceptible phenotypes. Only activity to 1-naphtholic esters unambiguously discriminated between susceptible and resistant aphids.

Field and Devonshire (1992) reported that the amplified esterase genes responsible for insecticide resistance in the aphid *Myzus persicae*. They were found only two restriction patterns, each associated with the presence of a different amplified esterase (E4 or FE4).

Liu *et al.* (1992) collected *Bemisia tabaci* from crops grown in desert regions of California (which included cotton, sweet potato and broccoli) represented a mixture of biotypes. One biotype was identical to a colony originally obtained in 1981 and since maintained in the laboratory. The other and most prevalent biotype could not be distinguished morphologically but could be distinguished by esterase isoenzyme banding patterns. The banding patterns of the biotypes were not affected by rearing on different plant species. Different stages of development and adults of both sexes had the same isoenzyme patterns.

Costa *et al.* (1993) analyzed nonspecific esterase banding patterns of the sweet potato whitefly, *Bemisia tabaci* (Gennadius), by native PAGE. Banding patterns of >300 *B. tabaci* adults from 30 populations in Hawaii matched the B type esterase banding pattern produced by the B biotype standard from Arizona. Banding patterns representative of other *B. tabaci* biotypes were not found.

Cuany *et al.* (1993) examined the action of B1 on chloropyrifos in organophosphate-resistant *Culex* mosquitoes. Esterase B1 activity in *C. pipiens* mosquitoes was strongly inhibited by oxidized organophosphates (OP), but not by nonoxidized forms or by carbamates. Inhibition by chloropyrifos oxon and paraoxon

remained total during the 2 hr. following the removal of free insecticide molecules, indicating that hydrolysis by esterase B1 was either very slow or absent. This hypothesis was confirmed by comparing the fate of [^{14}C] chloropyrifos in larvae of strians TEM-R (with the over produced esterase B1) and MSE (lacking an over produced esterase). As expected, large quantities of chloropyrifos oxon were observed in the two strains, but on other metabolite was found in TEM-R. It was concluded that esterase B1 confers resistance to at least diethyl OPs through sequestering rather than metabolism, as was also the case with the overproduced esterase E4 of *Myzus persicae*.

Bloch and Wool (1994) induced artificial selection for increased resistance to methidathion in two replicate lines of the whitefly, *Bemisia tabaci*. Esterase activity (measured from the hydrolysis of beta-naphthyl butyrate) increased in the selected lines in correlation with resistance. They observed no change in mean esterase activity in the unselected (control) population.

Moores *et al.* (1994) showed that the acetylcholinesterase in some resistant strains of *Myzus persicae* and its extremely close relative *Myzus nicotianae* marked insensitivity to inhibition by the established carbamate pirimicarb (>100-fold) and by triazamate, a

novel triazole aphicide (>10-fold) that acts on the same target. There was no insensitivity to a range of other carbamate and organophosphorous insecticides. This resistance mechanism appears to be rare at present and was only found in a heterozygous form associated with the commonly occurring elevated E4/FE4 esterases that confer broad cross-resistance to many aphicides. This insensitive target site mechanism, even when heterozygous, enhances the esterase-based resistance to pirimicarb and triazamate by 15- to 30-fold.

Ono *et al.* (1994) reported that Para Oxon is a potent inhibitor of greenbug esterases, and the resistance mechanism apparently involves rapid and tight binding of enzyme and insecticide that prevents the insecticide from reaching its target site. Native polyacrylamide gel electrophoresis also supported this view since staining of enhanced bands associated with resistance was inhibited by incubation with paraoxon. The resistance strains apparently produce more of the isozymes that are inhibited by paraoxon and are able to sequester a greater number of paraoxon molecules than susceptible greenbugs.

Sakata and Miyata (1994) observed several forms of carboxylesterase in a malathion-resistant small brown planthopper,

Laodelphax striatellus, by isoelectrofocusing (IEF). The mechanisms of increased esterase activity, esterases were purified and their biochemical characteristics were investigated in five active esterase isozymes of two resistant strains. These esterases have polymorphic characteristics and their molecular weights ranged from 66 to 70 kDa, due to variations in glycosylation. The pI values of these esterases ranged from 5.3 to 4.7. These esterases were immunologically related and NH_2-terminal amino acids were identical in all isozymes regardless of pI or molecular weight. No differences have been found in kinetic parameters (Km and Vmax) to alpha- naphthylacetate and specific activity toward alpha- naphthylacetate and malathion as a substrate in all isozymes. Resistant individuals showed high malathion carboxylesterase activity.

Moores *et al.* (1996) investigated the levels of insecticide resistance, total esterase activity; and acetylcholinesterase (AChE) sensitivity to inhibition by insecticides was investigated in three clones of *Aphis gossypii* Glover. Compared with LC50 values for a susceptible clone (171B), clone 968E showed high (>20-fold) resistance to most of the carbamate and organophosphorous insecticides tested, whereas clone 1081K only exhibited strong resistance to some carbamate insecticides. Aphids 171B had low esterase activity combined with an AChE variant showing baseline

sensitivity to organophosphorous and carbamate insecticides. Aphids 968E combined high esterase activity with broad-spectrum AChE insensitivity to these chemicals. Aphids 1081K had low esterase activity and an AChE variant that was insensitive to pirimicarb and a smaller range of OP insecticides.

Raymond *et al.* (1996) studied esterase polymorphism in insecticide susceptible populations of the mosquito *Culex pipiens* from various countries and found gene amplification involving a particular haplotype at the esterase B locus. This similarity has been explained by a unique amplification event followed by migration and selection by organophosphate (OP) insecticides. This assumes that the polymorphism of non-amplified esterase haplotype is so large that the chance of independent amplification in two distinct population is negligible.

Grafton-Cardwell *et al.* (1998) studied a population of California red scale, *Aonidiella aurantii* (Maskell) and found to be resistant to the organophosphates, chlorpyrifos and Methidathion, and to the carbamate carbaryl when compared with a susceptible population. The esterase inhibitor S, S, S,- tributyl phosphorotrithioate increased chlorpyrifos toxicity in the resistant population to a greater extent than in the susceptible population,

suggesting that esterase enzymes were involved in chlorpyrifos, but not methidathion or carbaryl resistance. The esterase banding patterns of both populations were characterized using cellulose acetate electrophoresis. As many as 5 esterase bands were detected in 2nd instar females, early 3rd instar non gravid females, late 3rd instar non gravid females, 2nd instar males, pupal males and adult males. There was variation between sexes and stages of scale in the relative mobility, frequency of occurrence and the activity of esterase bands. There was no consistent trend in the number of bands relative to insecticide resistance. However, ther was a trend in 5 of 6 scale stages tested for the insecticide-resistant population to exhibit higher esterase activity in band EST-3 compared with the susceptible population. When tested on the same cellulose acetate plate, insecticide-resistant, nongravid 3rd instar females exhibited significantly greater esterase activity in band EST-3 compared with susceptible individuals. When staining duration was reduced from 45 to 5 min, fewer esterase bands were detected; however, the EST-3 band remained and showed even greater differentiation in activity between susceptible and resistant individuals.

Hemingway *et al.* (1998) studied the role of gene splicing, gene amplify and regulation in mosquito insecticide resistance. In *Culex* mosquitoes, the most common organophosphate insecticide

resistance mechanism is caused by co-amplification of two esterases. The amplified esterases are differently regulated, with three times more Est $\beta2^1$ being produced than Est $\alpha2^1$. Cis-acting regulatory sequences associated with these esterases are under investigation. All the amplified esterases in different *Culex* sp. act through sequestration. The rates at which they bind with insecticides are more rapid than those for their non-amplified counter-parts in the insecticide-susceptible insects. In contrast, esterase, based organophosphate resistant in *Anopheles* is invariably based on changes in substrate specificites and increased turnover rates of a small subset of insecticides. The up-regulation of both glutathion S-transferases and monooxygenases in resistant mosquitoes is due to the effects of a single major gene in each case. The products of these major genes upregulate a broad range of enzymes. The diversity of glutathione S-transferases produced by *Anopheles* mosquitoes is increased by the splicing of different 5' ends of genes, with a single 3' end, within one class of this enzyme family.

Zhu and Gao (1999) compared biochemically the activity and sensitivity of acetylcholinesterase (AChE) from an organophosphate-susceptible (OSS) and three resistant strains of the greenbug (*Schizaphis graminum*). All three resistant strains displayed higher frequencies of individuals with respect to both increased activity of

were detected in nymphs. No genetic polymorphism has been detected thus far in the esterases of *Nasutitermes globiceps*. This study suggested that allozyme variation can be explored to understand social structure of this genus.

CHROMOSOMES

Demerec and Hoover (1936) were able, in the three stocks of *Drosophila* each with a different deficiency at one end of the X chromosome, to find approximately on the giant chromosomes, the location of the same genes found on the normal chromosome maps constructed by the percent of crossing over.

The ten long arms and the two dots of the salivary gland chromosomes of the fruit fly *Dacus oleae* (Gmel.) (Diptera: Tephritidae) were mapped for the first time. The mitotic chromosomes of that species were described. The affinities in chromosome morphology with the Medfly *Ceratitis capitata* (Wiedeman) were discussed (Krimbas, 1963).

The karyotype and chromosome behaviour of *Ceratitis capitata* was studied both in mitosis and meiosis by Radu *et al.* (1975). The chromosome number is 12 (n = 6), and includes two

pairs of metacentric chromosomes, two pairs of submetacentric and two pairs of subtelocentric chromosomes. They differentiated the sex chromosomes well and the X chromosome is typified by a secondary constriction.

As described by Southern (1976), intense C-banding occurs in centric blocks of all autosomes in *Ceratitis capitata*, in centric regions and the short arm of the X chromosome, and throughout the Y chromosome.

Foster *et al.* (1980) represented standard photographic maps of the trichogen cell polytene chromosomes. They determined locations of 39 mutations by genetic mapping of autosomal duplications, inversions and translocations.

Baimai *et al.* (1983) found that central-western Brazilian *D. serido* have metaphase plates, consisting of four pairs of telocentric autosomes, one pair of small dot 6^{th} chromosomes and one pair of sex chromosomes (X telocentric and Y acrocentric).

The Y chromosome of *Lucilia cuprina* was cytogenetically dissected by Bedo and Foster (1985). They recovered adjacent segregation products from crosses with appropiate autosomal and Y-

autosome translocations. By these means they isolated Y chromosomes lacking most of the short, long, or both arms. Only the centromeric portion of the Y chromosome was necessary for male determination and fertility, the bulk of the short and long arms having no role in sex determination. Additionally, it was shown that most of the short arm can be passed into the female line with no marked effect. These results, together with evidence from other studies, indicate that male determination in *L. cuprina* was centered in a discrete region near the Y chromosome centromere.

Beckenbach and Prevosti (1986) distinguished from the endemic *obscura*- subgroup species, *Drosophila pseudoobscura*, *D. persimilis*, *D. miranda*, by sex comb, vaginal plate and wing bristle morphology, by allozymic differences and by differences in the polytene chromosomes.

Robinson *et al.* (1986) developed a genetic sexing system using the ADH locus in the Medfly *C. capitata*, which was based on the sensitivity of ADH null mutations to environmental ethanol. A series of null mutants have been induced at this locus, however, none proved viable as homozygotes. One of these null mutants was translocated to the male determining chromosome and this line can be used for genetic sexing. It is proposed that the higher ADH activity of

the females (homozygous positive) in comparison with the males (heterozygous null) is responsible for their lower survival in larval medium containing allyl alcohol. ADH converts the allyl alcohol to the lethal ketone. The possible use of this line to sex large populations of medflies for use in sterile insect release programmes was discussed. ADH was mapped in the autosome 2.

As reported by Bedo (1986 and 1987), well banded polytene chromosomes were found in trichogen cells of the spatulate superior orbital bristles of male pupae of *C. capitata*. Five banded chromosomes and a prominent heterochromatic network associated with a nucleolus were found. She demonstrated that the five autosomes (Q-banded) are represented by conventional polytene chromosomes, while the sex chromosomes are contained in the heterochromatic net, most of which fluoresces strongly. In 1987, She presented photographic maps of orbital trichogen polytene chromosomes of *C. capitata* and established homologies between two chromosomes of the polytene and mitotic complements. She also presents measurements of the mitotic chromosomes and compares the banding patterns of trichogen and salivary gland chromosomes.

Wallace and Newton (1987) noted that in interphase cells of *Aedes aegypti* (L.) (2n = 4 + XX/ XY), only the nucleolus responded

to selective silver staining. The secondary constriction on the chromosome 3 remained unresponsive at all times but the six centromeres were identified throughout mitosis from early prophase as well as those stages of meiosis subsequent to diplotene. The centromeric blocks were not synonymous with the pericentric heterochromatin revealed by C-banding. X chromosome without an intercalary C-band were newly discovered in *Ae. aegypti* in the Bangalore strain. Sequential Q- or Hoechst 33258/ C-banding in this and Trinidad-30 strain revealed intercalary heterochromatin diversity within and between strains and also differences between intercalary an pericentric heterochromatin.

Zacharopoulou (1987) provided a photographic representation of the polytene chromosomes from the salivary gland of *Ceratitis capitata*, and some important landmarks were recognized in each arm. She described an XX / XY pair and five pairs of autosomes in the metaphases, but X and Y chromosomes were absent among the banded polytene chromosomes. She also constructed polytene chromosome maps of the five autosomes from salivary gland cells in the same species in 1990. She established the correlation of the polytene elements to mitotic chromosomes and linkage groups by using various Y-autosome and autosome-autosome translocation lines.

Kerremans *et al.* (1990) analyzed polytene chromosomes of three genetic sexing strains of *C. capitata*. The genetic sexing mechanism was based on a pupal colour dimorphism (white-brown) and was the result of a reciprocal translocation between the Y chromosome and the autosome bearing the w locus (white pupal case). The analyzed polytene chromosomes were derived from two different pupal tissues, the orbital bristle and fat body cells. The Y chromosome was visible in both tissues, while the autosomes present a different banding pattern. Based on these features, the autosome breakpoints in the three Y; autosome translocations were mapped and the homology of the translocated autosome in both tissues was established. In addition, the location of the break-points was compared to the stability of these three strains.

Six natural populations (three urban and three rural) of *Drosophila melanogaster* from India were analyzed for chromosome inversions, revealing the presence of 19 different paracentric autosomal inversions. One new inversion was also been detected in a laboratory stock established from flies collected from Kerala. In total 20 different paracentric inversions in India *D. melanogaster* have been detected, and of these 4 are common cosmopolitans; 2 are rare cosmopolitans; 7 are recurrent endemics; and 7 are unique endemics.

The quantitative data clearly show that the urban populations are different from rural ones with respect to inversion polymorphism (Singh and Das, 1990).

In 1990, Das and Singh analyzed ten laboratory stocks of *Drosophila melanogaster* initiated from females collected in different localities in India for chromosome inversions. Six inversions were found, three in 2L, one in 2R, one in 3L and one in 3R. Out of these six inversions, three were new and were being reported for the first time. The persistence of inversion polymorphism of *D. melanogaster* which were maintained for more than one year under laboratory conditions, suggested some advantages of inversion heterozygotes.

Zacharopoulou *et al.* (1991) analyzed cytogenetically a genetic sexing strain of *C. capitata*, carrying a null mutation for ADH activity linked to the Y chromosome. In addition to an insertion of a large part of the Y chromosome into chromosome 2, this strain carried two other chromosomal rearrangements, a deletion in the second chromosome and a reciprocal trnslocation involving chromosomes 2 and 4. The progeny of the T (2: 4) translocation heterozygote with unbalanced chromosomal constitution can survive upto the larval and (or) to the adult stage. These cytological

characteristics were discussed in relation to the genetic sexing behaviour of this line.

Zacharopoulou *et al.* (1992) hybridized cloned DNA segments to salivary gland polytene chromosomes of the Medfly, *C. capitata*, and thus established molecular markers for 24 sites on 6 out of 10 autosomal arms. An additional marker identified a Medfly repetitive element that hybridizes to approximately 100 autosomal sites as well as a granular network that was thought to represent the X chromosome. Some of the markers corresponded to 9 characterized transcription units, while 17 remained anonymous; at least 3 of the latter are restriction fragment length polymorphism (RFLP) markers. The characterized transcription units documented that chromosomal arm 5L of *C. capitata* was homologous to the *Drosophila melanogaster* X chromosome.

The recent investigation of Mavragani-Tsipidou *et al.* (1992) made the first attempt to construct a photographic map of the larval fat body polytene chromosomes of *Dacus oleae*. In addition, the mitotic karyotypes of brain ganglia were examined, permitting tentative correlations between mitotic and polytene elements.

A chromosomal polymorphism affecting the length of the long arm of the X-chromosome, along with the presence of a B-chromosome was reported (Basso and Lifschitz, 1995), in a laboratory population of *C. capitata*. The observed B-chromosome was small, heterochromatic and telocentric. It was found, in both sexes, in somatic cells (cerebral ganglia tissue) as a free chromosome, or terminally attached to the long arm of the X-chromosome, giving the appearance of a larger X-chromosome. Males transmitted both free B and large X-chromosomes to their progeny. Females only transmitted large X to their progeny.

Basso *et al.* (1995) identified two types of naturally occurring Y chromosome in laboratory stocks of the Medfly, *C. capitata*, viz., the standard Y chromosome Ya and a shorter Y chromosome Yb. The C-banding technique applied to this material has revealed an intercalary band on the long arm of the Ya. This distinctive banding pattern was described as a reliable means to determine sexual chromosome polymorphisms.

Zambetaki *et al.* (1995) presented photomaps of the malpighian tubules and the salivary gland polytene chromosomes of *Bactrocera oleae* (*Dacus oleae*) and compared with those of the fat body. They observed a heterochromatic mass corresponding to the six

chromosomes in the nuclei of the three somatic tissues. They also noted the reverse tandem duplications, as well as the rather unusual ectopic pairing of the telomeric regions of different chromosome arms. In addition, the constancy of the banding pattern based on the analysis of the three larval tissues was discussed.

A complementary DNA clone derived from the Medfly white gene was isolated (Zwiebel *et al.*, 1995), which showed substantial similarity to white genes in *Drosophila melanogaster* and other Diptera. It was correlated with spontaneous mutation causing white eyes in the Medfly and could be used to restore partial eye colour in transgenic *Drosophila* carrying a null mutation in the endogenous white gene.

Baimai *et al.* (1995) focused their study on different forms of mitotic karyotypes of the wild samples of the *Bactrocera dorsalis* complex, particularly on chromosomal differentiation that was due to the different amount and distribution of major block(s) of constitutive heterochromatin. They showed that chromosomal evolution among closely related species within the *B. dorsalis* complex clearly involves the presence or absence of constitutive heterochromatin in the centromeric regions of autosomes as well as in the X chromosome. Baimai *et al.* (1996) also reported the results of

analysis and comparison of mitotic karyotypes of five species of *Bactrocera* with special emphasis on quantitative differentiation and distribution of constitutive heterochromatin in the genome.

Hadi *et al.* (1995 and 1996) studied larval polytene chromosomes on the six species of the blackfly, *Simulium*. In 1995, they observed and mapped three pairs of polytene chromosomes in *S. yaeyamaense*. Chromosome I is metacentric and the two shorter pairs, chromosomes II and III were submetacentric. The centromeric region of all chromosomes was not expanded and barely recognized by the existence of a heavy band. They recognized nucleolar organizer, Balbiani ring, double bubble, and Parabalbiani ring. They examined from three populations, one inversion (III-1) was always heterozygous in females, and four other inversions (III-2, III-3, III-4 and III-5) were polymorphic and found as heterozygote in both sexes. In 1996, standard maps of larval salivary gland polytene chromosomes of three Japanese blackfly species, i.e., *S. bidentatum*, *S. aokii* and *S. arakawae* were presented by them. They found a Balbiani ring, double bubble, a Parabalbiani ring and a nucleolar organizer on the same arm of the same chromosome. The precise locations of these main characteristics were different among the species. In *S. bidentatum* and *S. aokii* polymorphic inversions were found in low frequencies, and B-chromosomes were also detected. In

all chromosomes of *S. bidentatum*, centromere consisted of large conspicuous heterochromatic segment. Five inversions were found and none of them was sex related. C-band staining successfully revealed that band of chromosome III, was heterozygously stained, slightly and heavily stained in males, while homozygously stained, only heavily in females. In this way they ascribed the slightly stained band to Y-chromosomal, and the heavily stained, to X-chromosomal. In addition, they noted a haplotype on *S. eximium* involving two distinct heterochromatic bands in the centromere (21A) and section 20B of the chromosome was heterozygous in most of the males from the populations of Puncak (West Java) and appeared to be sex-linked.

The metaphase and salivary gland chromosomes of *Drosophila seriema* were investigated from the samples of seven different localities, which cover the known geographical range of the species. Very little chromosomal variability was found. A new karyotype was observed in two different localities and, in addition, the fifth chromosome was found to be polymorphic for a small paracentric inversion in one of the stocks. Thus, that species appeared to be monomorphic, with low interpopulational differentiation, at least at the chromosomal level, in comparison with other species of the *buzzatii* complex. The phylogenetic relationships of *D. seriema* with its nearest relatives were discussed. In addition, they analyzed

the polytene chromosomes of the hybrid larvae from the cross between *D. seriema* and *D. borborema*. Thus allowed a direct comparison of the chromosomal arrangements between these two species (Kuhn *et al.*, 1996).

Polytene chromosomes of *Chironomus tentans* from Europe, Siberia, and North America were examined to clarify genetic relationships among widely distributed populations of the Holarctic midge. The first extensive cytogenetic analysis of Siberian populations confirmed earlier suppositions that *Chironomus tentans* karyotypes were quite uniform and two inversion sequences were found in the 18 Siberian population examined. Of the 19 sequences found in the three American populations studied, only 6 were share with the Palearctic. Three of the seven chromosome arms in Nearctic *Chironomus tentans* had no sequences in common with European populations and four shared none with Siberian population (Kiknadze *et al.*, 1996).

The sex chromosomes of the tephritid fruit fly *Ceratitis capitata* (Wiedeman) were heteromorphic. The male-determining region was located on the Y chromosome by deletion mapping using unbalanced offspring from several translocation strains. Only 15% of the Y chromosome was required for male determination and male

fertility. Based on this result, Willhoeft and Franz (1996) expected to find Y-chromosomal length polymorphism in natural populations. Using fluorescence in situ hybridization with two repetitive DNA probes that label the Y chromosome, no obvious size difference were detected in seven wild type strains and three mutant strains. They also analyzed two wild-type strains established recently from pupae sampled in Kenya. The Y chromosome showed a polymorphism in the hybridization pattern of a repetitive Y-specific Medfly clone. However, the overall size of the Y chromosome was similar to that of the other strains. Besides karyotypes of *Ceratitis capitata*, *C. rosa* and *Trirhithrum coffeae* were analyzed (also in 1996) by C-banding. Furthermore, the ribosomal genes were mapped to the sex chromosomes in these species. In the same year they mapped the region containing the relevant factor through the analysis of Y-autosome translocations using fluorescence *in situ* hybridization with two different probes.

Shahjahan *et al.* (2000) were observed larval salivary gland polytene chromosomes of the melon fly *Bactrocera cucurbitae* from laboratory reared stocks. In their study, attempts were made to construct a diagrammatic sketch of the polytene chromosomes of this species. This species had five pairs of polytene chromosomes which were numbered I-V according to their gross size. Several dark bands

(ILB) and a group of dense bands (IRB) were found in chromosome I. Chromosome II was recognized by a compact dark band (IILA), three light bands (IIRA) and three successive pairs of dark bands (IIRB). Chromosome III was characterized by the presence of different bulbs and puffs in different regions. Chromosome IV possesses three successive dark bands (IVLA) and a swollen tip (IVRA). Chromosome V was recognized by different bulbs and puffs in the marker regions (VLA, VLB, VRB).

Shahjahan *et al.* (2002) reported the salivary gland chromosome analysis in *B. cucurbitae*. A photographic representation of the polytene genome of this species is made where the banding pattern, identification as well as some important landmarks are recognized in each arm. Like other tephritids this species has five pairs of salivary gland chromosomes which were numbered from I-V according to diagnostic features and landmarks at each chromosome arm.

According to Shahjahan and Yesmin (2002), standard photographic maps of the polytene chromosomes are presented for the melon fly *Bactrocera cucurbitae*, a serious pest of fleshy fruits and vegetables. Five larval salivary gland polytene chromosomes (10 polytene arms) were isolated, and their characteristic features and

landmarks have been recognized. Banding patterns of each of the polytene arms are presented., where variation in band intensity and puffs appear to reflect fundamental differences in chromosomes. The whole polytene genome has been typically mapped by dividing it into 100 sections and the subsections were lettered. The mitotic chromosomes of larval brain ganglia were also examined, five pairs of autosomes and an XX/XY sex chromosome pair. In addition, a heterochromatic mass corresponding to the sex chromosomes were observed in the polytene nuclei of salivary gland tissue. This investigation showed that *B. cucurbitae* has excellent cytological material for polytene chromosome analysis and proved to be very useful for obtaining more detailed genetic information on the pest's natural populations.

Chapter-3

Materials and Methods

3.1 FLY STOCKS

Different life stages of the melon fly, *B. cucurbitae* originating from a laboratory-reared stock were used for the observation of esterase isozyme variation. The larvae (1st and 2nd instar) were also used for the construction of photographic polytene chromosome maps. This population has been maintained in the fruit fly laboratory, Radiation Entomology and Acarology Division, IFRB, AERE, Savar, Dhaka for more than 6 years and has also been cultured the same laboratory, still up to date.

3.2 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

3.2.1 Introduction

A technique which separates the dissolved particles subjected to an electrical field, based on their Relative Mobility (RM) values, is called electrophoresis (Strickberger, 1996). First used by Lewontin and Hubby (1966), the technique of gel electrophoresis has subsequently been widely applied to the study of natural populations. By using it, scientists have been discovered a truly remarkable degree of polymorphism in these populations (Raven and Johnson, 1986).

Acrylamide gel has become extremely useful in electrophoresis. It offers a better method to control pore size of the gel, leading to clearer and sharper banding patterns in some instances (Steiner and Joslyn, 1979). Besides, it is chemically inert, stable over a wide range of pH, temperature, and ionic strength, and is also transparent. For these and other reasons polyacrylamide gels have become the medium of choice for zone electrophoresis of most proteins for the analysis of isoenzymes (Hames, 1986).

For the purpose of present study, PAGE of melon fly homogenates was performed in a vertical system by using polyacrylamide slab gels and continuous non-dissociating buffer system as described by Hames (1986), with some modifications. The occurrence of possible epigenetic bands was prevented by the presence of EDTA in the gel buffer (Callaghan *et al.*, 1994). The techniques are as follows:

3.2.2 Apparatus

Zone electrophoresis of proteins in polyacrylamide slab gels is almost always now carried out in vertical format (Hames, 1986). List of the apparatus has been presented below:

1. Centrifuge Machine, Kubota 1300 (Kubota Corp., Japan).
2. Electrophoresis units (Osaka, Japan)

3. Power packs:

   i. Volt line (Griffin) for smaller electrophoresis unit.

   ii. Pharmacia LKB-ECPS 3000/150 for larger unit.

4. Electronic balancer, AA 160 (Denver Instrument Company).

5. Stirrer, HB502 (Bibby Sterlin Ltd., UK).

6. Digital pH meter (Institute of Electronics, Bangladesh Atomic Energy Commission).

7. Water bath (Gallen Kamp).

8. Incubator (Memmart GTR0214, Germany).

9. Micropipette (10 and 50 µl)

3.2.3 METHODS

3.2.3.1 Preparation of Stock Solutions

The solutions, which are not stable for long time, were made fresh just before use.

The stock solutions prepared are as follows:

1. Acrylamide- bisacrylamide (29.1: 0.9) stock solution:

   - This solution was prepared by dissolving 29.1 g of acrylamide and 0.9 g bisacrylamide in 70 ml distilled water.
   - After stirring, the total volume was made 100 ml.

- The solution was filtered through Whatman No. 1 filter paper and stored at 4^0C in a dark bottle.
- This stock solution was made for one week only.

2. Tetramethyl ethelene diamine (TEMED): TEMED was used as supplied. It was kept at 4^0C in a dark bottle.

3. Ammonium per sulphate (10%): Ammonium per sulphate (0.1 g) was dissolved in 1 ml of distilled water. As this solution is unstable it was always made fresh just before use.

4. Electrophoretic buffer:

Tris-Borate (pH 8.9) – Concentration 5X solution

- 54 g of Tris base and 27.5 g Boric acid were dissolved in 500 ml of water.
- 20 ml of 0.5 M EDTA was added to the solution.
- After stirring the solution on a magnetic stirrer, the total volume was made upto 1 litre.
- This 5X buffer stock was stored at room temperature and used for not more than 1 week.

5. Sample loading buffer (2X):

2X sample loading buffer solution was prepared by dissolving 0.83 g bromophenol blue and 1.33 g sucrose in a total volume of 10 ml water. It was stored at 4^0C for later use.

6. Staining buffer:

a. NaH_2PO_4 (0.2 M Monobasic sodium phosphate): It was prepared by dissolving 2.64 g Monobasic sodium phosphate in 100 ml water.

b. Na_2HPO_4 (0.2 M Dibasic sodium phosphate): It was prepared by dissolving 1.07 g Dibasic sodium phosphate in 20 ml water.

To make the final buffer (pH 6.4), 100 ml of 0.2M Monobasic sodium phosphate was mixed with 20 ml 0.2M Dibasic sodium phosphate. This buffer was always prepared fresh before use.

7. Substrate mixture: The substrate mixture was always prepared fresh just before use.

α-naphthyl acetate (Esterase substrate- black bands).

- 0.09 g α-naphthyl acetate was dissolved in 2 ml acetone and 2 ml water.

β-naphthyl acetate (Esterase substrate- red bands)

- 0.09 β-naphthyl acetate was dissolved in 4 ml acetone.

Finally, 4 ml α-naphthyl acetate solution and 4 ml β-naphthyl acetate solution were added to 120 ml of staining buffer (6). This solution was known as substrate mixture.

8. Staining mixture (Fast blue RR salt):

Staining mixture was prepared by dissolving 0.10 g Fast blue RR salt in 150 ml of water just before use.

9. Washing solution:

Washing solution was prepared as follows:

- 80 ml of methanol and 14 ml of glacial acetic acid was measured with measuring cylinder.
- These were mixed with distilled water in a beaker to make the final volume 200 ml.

3.2.3.2Preparation of Gel

During the first day of this work, 5% gel was prepared for electrophoresis and used constantly since it gave the better

separations of enzymes. Here gel recipe for 5% polyacrylamide gel is presented in the following **Table**, which was prepared by using the standard protocol (Shahjahan *et al.*, 2001 and 1998), with some modifications.

Table. Recipe for Gel preparation using Non-dissociating continuous buffer system.

Stock solution	**Volumes (ml) of the various reagents to make 22 ml of gel mixtures**
Acrylamide-bisacrylamide (29.1: 0.9)	5 ml
Continuous buffer (1X Concentration) of TBE	6 ml
Ammonium per sulphate (AMPS)- 10%	250 µl
Distilled Water	10.50 ml
TEMED	250 µl

Procedure

1. Cleaning the glass plates: The gel plates were perfectly cleaned to obtain good gel adhesion into the glass. Soaking them in chromic acid overnight, rinsed in water, and then with ethanol cleaned the glass plates. Then the plates were put down onto clean tissue paper, with the side, which was to be in

contact with the gel upper most, and swabbed with an acetone-soaked tissue held in a gloved hand. After final rinse with ethanol, the plates were allowed to air dry.

2. Assembling of glass plates: A rubber cord was positioned between two glass plates at the bottom very carefully so that the plate bottom is sealed precisely to prevent leakage of gel mixture. Then, the plate assembly is clamped together with strong metal clips, which were positioned to press on the sandwich just over the spacer positions at right and left edges, and over the rubber cord position at the plate bottom.

3. Pouring the gel mixture: The clamped plate assembly was held at an angle leaning against a supporting object during pouring the gel. The gel mixture was prepared by adding the correct volumes of all components, except AMPS, to a small thick-walled beaker. The AMPS was added, gently mixed in, and the gel solution was then poured without delay between the glass plates. Immediately, the comb was inserted between the glass plates and into the gel mixture. Special care was given to ensure that the teeth of the comb were fitted snugly against the glass plates, and that air bubbles were not trapped beneath the

comb otherwise irregularly shaped sample wells would be formed.

4. Polymerisation of the gel: The assembly was left undisturbed for the gel to polymerise (10-30 min), as evidenced by the appearance of a sharp boundary below areas of gel/air interfaces. After another 10 minutes, the comb was removed carefully and slowly.

5. Cleaning the gel wells: The sample wells formed by removal of the comb was rinsed out with reservoir buffer and then filled with the same buffer. If the gel was to be stored before use, the sample wells were rinsed out and the gel was kept in reservoir buffer or buffer of the same composition as in the gel.

3.2.3.3 Preparation of Sample

(a) Each sample was taken into an eppendorf tube and squashed in 100 µl of 1X Tris-Borate- EDTA buffer.

(b) Another 50 µl of Bromophenol blue solution was added to each sample.

(c) The samples were then centrifuged at 13000 rotation per minute (rpm) for 25 minutes at 4^{0}C.

(d) Supernatant was used for electrophoresis.

3.2.3.4 Sample Loading and Electrophoresis

During electrophoresis, using slab gels for the notched glass-plate system, the gel sandwich is attached to the electrophoresis apparatus before sample loading. A pre-electrophoresis preceded sample loading. Sample loading and electrophoresis procedure was as described below:

1. Rubber cord, metal clips, etc. used for gel preparation were removed, from the gel sandwich. Wells were washed by buffer solution. Reservoir buffer was added to the lower reservoir of the electrophoresis apparatus. The sandwich was placed on the electrophoresis tank. Essentially special care was given to remove any air bubble from the bottom of the gel, otherwise these would prevent uniform electrical contact between gel and reservoir buffer. Then the gels, prepared in notched glass-plate sandwiches, were clamped in position using metal clips with the notch of the glass-plate aligned with the notch in the upper reservoir.

2. The upper reservoir was filled with electrode buffer.

3. The apparatus was connected to the power pack (switched off) with the anode connected to the bottom reservoir, and the cathode connected to the upper reservoir. The power pack was then connected to the mains, switched on, adjusted to deliver 120 volts constantly. Thus a prerun was performed for about 30 minutes.

4. After prerun, the power pack was switched off and preferably the apparatus was disconnected from the pack.

5. The samples were then carefully loaded onto the wells using a micropipette, one sample to one well. The tip of the micropipette was held at an angle just inside the well to minimise sample mixing with the reservoir buffer.

6. Again the apparatus was connected to the power pack which was then switched on. Thus, running was going on at 120v constant voltage. In this condition, the electrophoresis apparatus was kept undisturbed for at

least three hours or until the tracking dye (bromophenol blue) was reached the gel bottom.

3.2.3.5 Recovery of Gels

Recovery of gels was another difficult work. A little lack of care during recovery of gels might result into torn-out gels. After completion of electrophoresis, gel was recovered from the gel cassettes/ glass-plates sinking in water very cautiously. First the notched glass plate was detached and, then the gel was recovered from other glass plate.

3.2.3.6 Staining

The gel was taken to a staining tray and 120 ml of substrate mixture was poured onto it. It (gel) was kept in this mixture for 15 minutes at 25^0C. After 15 minutes, substrate mixture was out poured and 150 ml of fast blue solution was added to the gel. In this condition, the gel was kept in incubator at 37^0C for 40 minutes.

3.2.3.7 Washing

After staining, fast blue solution was drained out and the gel was kept in washing solution overnight.

3.2.3.8 Preservation of Gels and Photography

The gel was then stored in TBE (1X) buffer solution at room temperature for later observation. A zoom lens camera took photograph of the gel, to put the gel on a white background.

3.2.3.9 Numbering of Esterase Isozymes

In the scanned photographs, positive and negative poles have been marked by positive (+) and negative (-) signs. The electrophoretically separated bands of esterase isozymes were numbered from the anodal end of the gel according to the recommendations of the Standard Committee of Enzyme (Webb, 1964).

3.2.3.10 Measurements of Relative Mobility

The value of most mobile band was considered as one when the relative mobility values of different bands of esterase isozyme were measured (Shahjahan *et al.*, 2001). According to previous Mendelian inheritance studies on these esterase loci (de Stordeur, 1976), each band corresponded to one allele.

(v) Microscope slides

(vi) Cavity slides

(vii) Cover slips

(viii) Razor

3.3.1.3 Chemical Agents

(i) 45% Acetic acid

(ii) 3N HCl

(iii) Lacto acetic acid

(iv) Lacto acetic orcein

(v) Synthetic orcein

(vi) Glacial acetic acid

(vii) Lactic acid

3.3.2 METHODS

3.3.2.1 Preparation of Chemical Agents

(i) 45% acetic acid: 45 ml glacial acetic acid to 55 ml distilled water.

(ii) 3N HCl: 3 ml HCl (assay 36.6%) to 7 ml distilled water.

(iii) Lacto acetic acid: 1: 2 (lactic acid- 60% acetic acid).

(iv) Lacto acetic orcein solution: 100 cc of the lacto acetic orcein solution was made by dissolving 2 g of synthetic

orcein into 50 cc of hot glacial acetic acid, adding 50 cc of 85% lactic acid after removing from heat. Then the solution was filtered by filter paper (whatman 1), inserted in a funnel, and the prepared solution was preserved in an incubator (-10 to 50°C). Glacial acetic acid was hotted, by using the magnetic stirrer with hot plate.

All the weightable and liquid chemicals were measured by the electronic balance and measuring cylinders, respectively.

3.3.2.2 Preparation of Polytene Chromosomes

Larvae of 1st instar (36 hrs old) and 2nd instar (3-4 days old) were used for the salivary gland polytene chromosome preparations as described by Zacharopoulou (1987).

Larvae were dissected in 45% glacial acetic acid and the glands were fixed in 3N HCl for 3-5 min. They were then transferred to a drop of lacto-acetic acid (lactic acid-60% acetic acid, 1: 2) for 5 min. The glands were then stained in lacto-acetic orcein for about 30-60 min. Excess stain was removed by washing the glands two or three times in lacto-acetic acid before squashing. Phase-contrast

microscope is used to examine the polytene chromosome preparations using a 40x objective. Well spread nuclei were used for the construction of polytene chromosome photographs presented. Photographs that showed the best morphology for each chromosome region were selected and used for the composite chromosomes presented.

Chromosome maps were analysed using the conventions of Bedo (1977 & 1987) and Mavragani-Tsipidou *et al.* (1992). In this system the total polytene chromosome complement is assigned 100 sections and the chromosomes allocated a number of sections and section limits marked along the chromosome using, wherever possible, prominent or distinctive banding features as delimiters. After the section limits were assigned, subsections were marked, again using the more prominent features available within it.

3.3.2.3 Photography

A Nikon (Optiphot-2) phase contrast microscope with relevant photographic attachment was used to examine the polytene chromosome preparations and photographs were taken on Fuji 100 film.

Chapter-4

Observations and Results

OBSERVATIONS AND RESULTS

4.1 ELECTROPHORESIS

The electrophoretic banding patterns of esterase isozymes were assigned to increase the numbers based on decreasing mobility following Richardson *et al.* (1986) and Webb (1964). For example, Esterase-1 (EST-1) referred to the band with greatest mobility. Esterase-1 and Esterase-2 (EST-2) were studied in eggs, larvae, pupae, adults and also the larval food media, sweet gourd (SG). All the seven bands (**Fig. 1**) of two different esterase isozyme loci except Est-$2^{0.27}$ (**Table 3** and **Plate 4**) were found in the population.

4.1.1 ESTERASE ISOZYME VARIATIONS

Third instar (6 days old) larvae

Electrophoretic banding patterns of esterase isozymes in third instar larvae were observed on 5% Polyacrylamide Gel Electrophoresis (PAGE).

All tested third instar larvae demonstrated the presence of strongly stained and overproduced EST-1 and EST-2 bands. Electrophoretically distinct bands, with close but different mobilities.

at each locus, could not be separated. They often appeared as single broad band in each case of EST-1 and EST-2.

The most, weakly staining and most mobile esterase was found to be polymorphic. These bands were clear in the stained gel, but in the scanned photographs (**Plates 2, 3 and 4**) they did not appear clearly. Esterase patterns of these two loci have also been presented in **Tables (1, 2 and 3).**

Esterase-1 (EST-1)

A total of 56 3rd instar larvae were analysed for EST-1 by polyacrylamide gel electrophoresis (**Plates 2, 3 and 4**). All 56 individual possessed the most strongly staining overproduced Est-$1^{0.46}$ band. Thirteen individuals displayed only one band (Est-$1^{0.46}$), another 13 displayed two bands (Est-$1^{0.46}$ and Est-$1^{0.58}$), 17 displayed three bands (Est-$1^{0.46}$, Est-$1^{0.87}$ and Est-$1^{1.00}$) and 13 individuals displayed four bands (Est-$1^{0.46}$, Est-$1^{0.58}$, Est-$1^{0.87}$ and Est-$1^{1.00}$) at this locus (**Tables 1, 2 and 3**).

For the computation of allele frequencies, middle band of the three bands group was assumed to be additional intermediate band produced by heterozygotes, and removed. In case of four bands,

middle two bands were removed. Thus, the observed numbers and proportions of the phenotypes are presented in the **Table 5.**

Four alleles were scored for EST-1. As no null individual was found, it was assumed that no null allele was present in the population, and displaying only one band was the result of homozygosity. The allele frequencies obtained have been presented in the **Table 6.** Thus, Est-$1^{0.46}$ was in the highest frequency 61.61%, in the population. Next highest frequency, 26.79%, was found for the Est-$1^{1.00}$ allele and 11.61% for Est-$1^{0.58}$ allele.

Est-$1^{0.46}$ and Est-$1^{1.00}$ bands were with very high frequency, Est-$1^{0.58}$ and Est-$1^{0.87}$ bands were with comparatively less frequency. Presence of excessively active EST-1 bands with highest frequency indicates that an esterase overproduction resistance mechanism is present in the studied population.

Esterase-2 (EST-2)

A total of 56 3rd instar larvae were analysed for EST-2 isoalleles by polyacrylamide gel electrophoresis (**Plates 2, 3 and 4**). Null individual at this locus was not found, indicating that either null alleles were absent, or, if present, with a very low frequency in the population. For the computation of allele frequencies, it was assumed

that null allele was absent from this population. Thirteen individuals displayed all three bands (Est-$2^{0.17}$, Est-$2^{0.27}$ and Est-$2^{0.37}$) and another 43 individuals displayed two bands. Among 43 samples, Est-$2^{0.17}$ and Est-$2^{0.27}$ bands were observed in only 13 individuals, whereas, another 30 individuals displayed the bands of Est-$2^{0.17}$ and Est-$2^{0.37}$.

Three alleles were scored for EST-2 locus. Frequencies of the different alleles at this locus were calculated with the assumption that all the single band expressions were the result of homozygosity for the respective alleles. This provided the highest frequency for the Est-$2^{0.17}$ allele that was 50% at EST-2 locus. Next highest frequency, 38.39% was found for the Est-$2^{0.37}$ allele and 11.61% for Est-$2^{0.27}$ allele (**Table 6**).

Est-$2^{0.17}$ band was displayed with high frequency, indicating that an esterase overproduction resistance mechanism is also present in this population.

Developmental Stages of *B. cucurbitae*

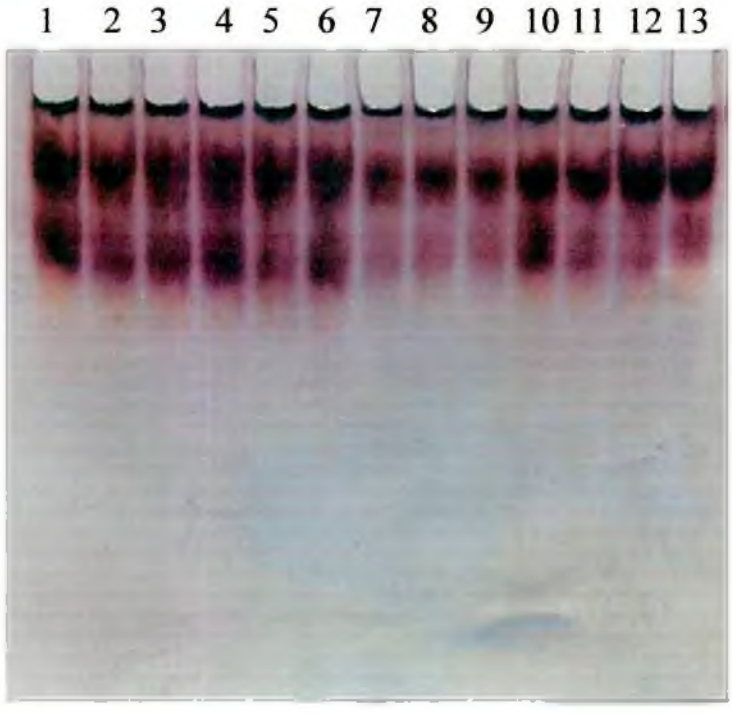
Esterase isozyme patterns were also found during the different life stages of *B. cucurbitae* by using 5% PAGE. Different electromorphs of EST-1 and EST-2 appeared as distinct bands in the different stages (i.e., larvae, pupae and adults) of the melon fly except

egg (**Plate 12**). Esterase banding patterns were scored from the stained gels, which are represented in the **Table 7**. Different stages of development and adults of both sexes had almost same isoenzyme patterns. But EST-1 and EST-2 bands appear to be sex-related since they were more intense in the females (**Plates 12 to 15**).

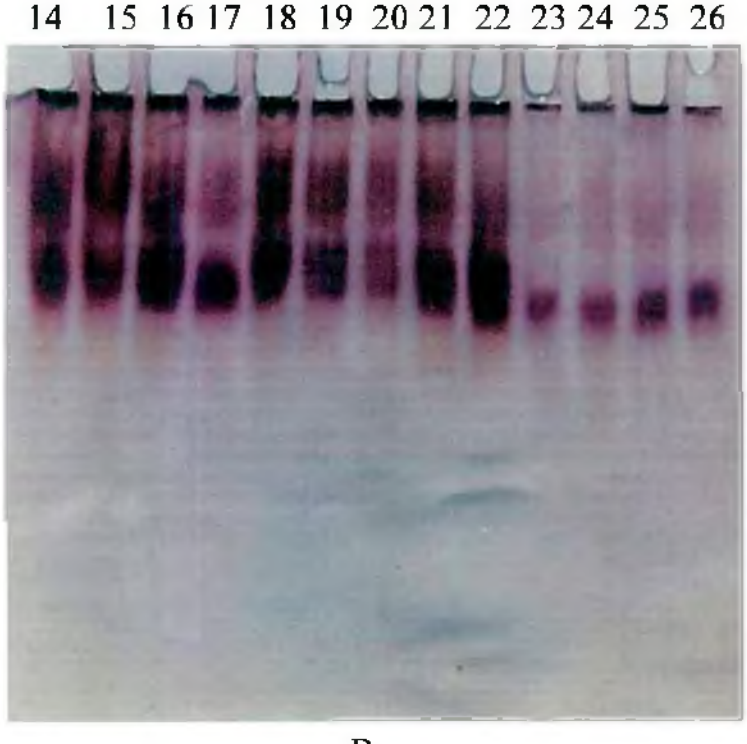
Table. 1. Esterase isozyme pattern* of third instar (6 days old) larvae of *B. cucurbitae* (+ = present and - = absent).

Slot Nos.	EST-1 bands				EST-2 bands		
	Est-$1^{1.00}$	Est-$1^{0.87}$	Est-$1^{0.58}$	Est-$1^{0.46}$	Est-$2^{0.39}$	Est-$2^{0.27}$	Est-$2^{0.17}$
1	-	-	-	+	+	+	+
2	-	-	-	+	+	+	+
3	-	-	-	+	+	+	+
4	-	-	-	+	+	+	+
5	-	-	-	+	+	+	+
6	-	-	-	+	+	+	+
7	-	-	-	+	+	+	+
8	-	-	-	+	+	+	+
9	-	-	-	+	+	+	+
10	-	-	-	+	+	+	+
11	-	-	-	+	+	+	+
12	-	-	-	+	+	+	+
13	-	-	-	+	+	+	+
14	-	-	+	+	-	+	+
15	-	-	+	+	-	+	+
16	-	-	+	+	-	+	+
17	-	-	+	+	-	+	+
18	-	-	+	+	-	+	+
19	-	-	+	+	-	+	+
20	-	-	+	+	-	+	+
21	-	-	+	+	-	+	+
22	-	-	+	+	-	+	+
23	-	-	+	+	-	+	+
24	-	-	+	+	-	+	+
25	-	-	+	+	-	+	+
26	-	-	+	+	-	+	+

* scored from stained gel.



A



B

Plate. 2. Electrophoretic banding patterns of esterase isozymes in third instar larvae (6 days old) of *B. cucurbitae* (Slot Nos. 1- 26).

Table. 2. Esterase isoyme pattern* of third instar (6 days old) larvae of *B. cucurbitae* (+ = present and - = absent).

Slot Nos.	EST-1 bands				EST-2 bands		
	Est-$1^{1.00}$	Est-$1^{0.87}$	Est-$1^{0.58}$	Est-$1^{0.46}$	Est-$2^{0.37}$	Est-$2^{0.27}$	Est-$2^{0.17}$
27	+	+	-	+	+	-	+
28	+	+	-	+	+	-	+
29	+	+	-	+	+	-	+
30	+	+	-	+	+	-	+
31	+	+	-	+	+	-	+
32	+	+	-	+	+	-	+
33	+	+	-	+	+	-	+
34	+	+	-	+	+	-	+
35	+	+	-	+	+	-	+
36	+	+	-	+	+	-	+
37	+	+	-	+	+	-	+
38	+	+	-	+	+	-	+
39	+	+	-	+	+	-	+
40	+	+	-	+	+	-	+
41	+	+	-	+	+	-	+
42	+	+	-	+	+	-	+
43	+	+	-	+	+	-	+

* scored from stained gel.

Table. 3. Esterase isoyme pattern* of third instar (6 days old) larvae of *B. cucurbitae* (+ = present and - = absent).

Slot Nos.	EST-1 bands				EST-2 bands		
	Est-$1^{1.00}$	Est-$1^{0.87}$	Est-$1^{0.58}$	Est-$1^{0.46}$	Est-$2^{0.37}$	Est-$2^{0.27}$	Est-$2^{0.17}$
44	+	+	+	+	+	-	+
45	+	+	+	+	+	-	+
46	+	+	+	+	+	-	+
47	+	+	+	+	+	-	+
48	+	+	+	+	+	-	+
49	+	+	+	+	+	-	+
50	+	+	+	+	+	-	+
51	+	+	+	+	+	-	+
52	+	+	+	+	+	-	+
53	+	+	+	+	+	-	+
54	+	+	+	+	+	-	+
55	+	+	+	+	+	-	+
56	+	+	+	+	+	-	+

* scored from stained gel.

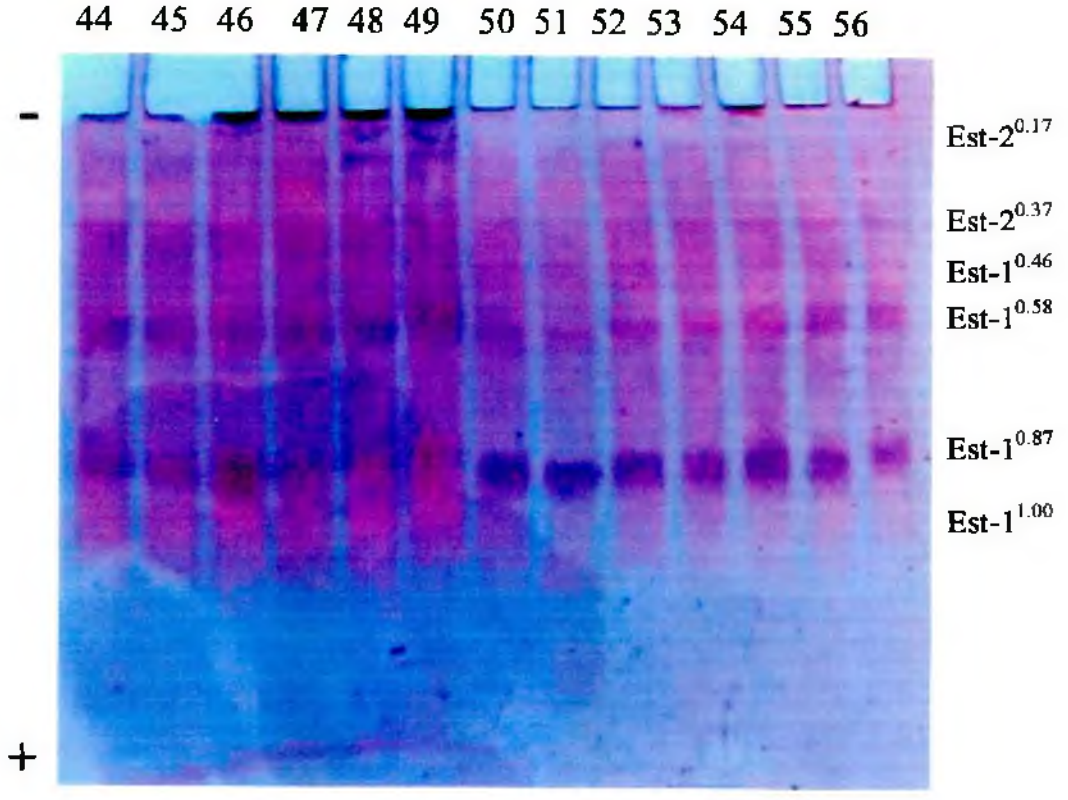


Plate. 4. Electrophoretic banding patterns of esterase isozymes in third instar larvae (6 days old) of *B. cucurbitae* (Slot Nos. 44- 56).

Table. 4. Total observed numbers of seven esterase bands in 56 individual of third instar larvae. These bands are controlled by two esterase isozyme loci on the basis of their relative mobility (RM) values.

Esterase isozymes	**Mean RM values**	**Total observed numbers**
EST-1	Est-$1^{1.00}$	30
	Est-$1^{0.87}$	30
	Est-$1^{0.58}$	26
	Est-$1^{0.46}$	56
EST-2	Est-$2^{0.37}$	43
	Est-$2^{0.27}$	26
	Est-$2^{0.17}$	56

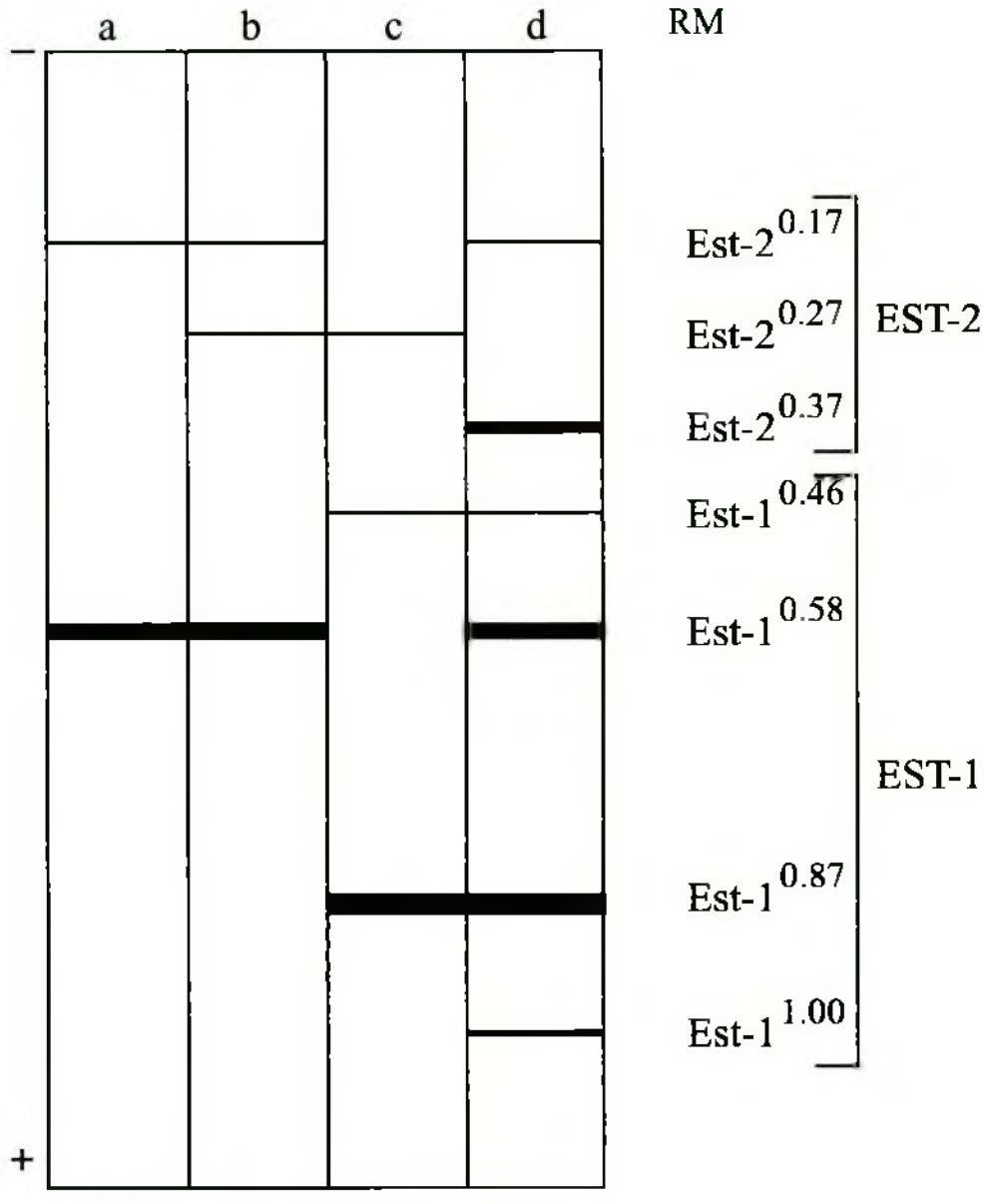


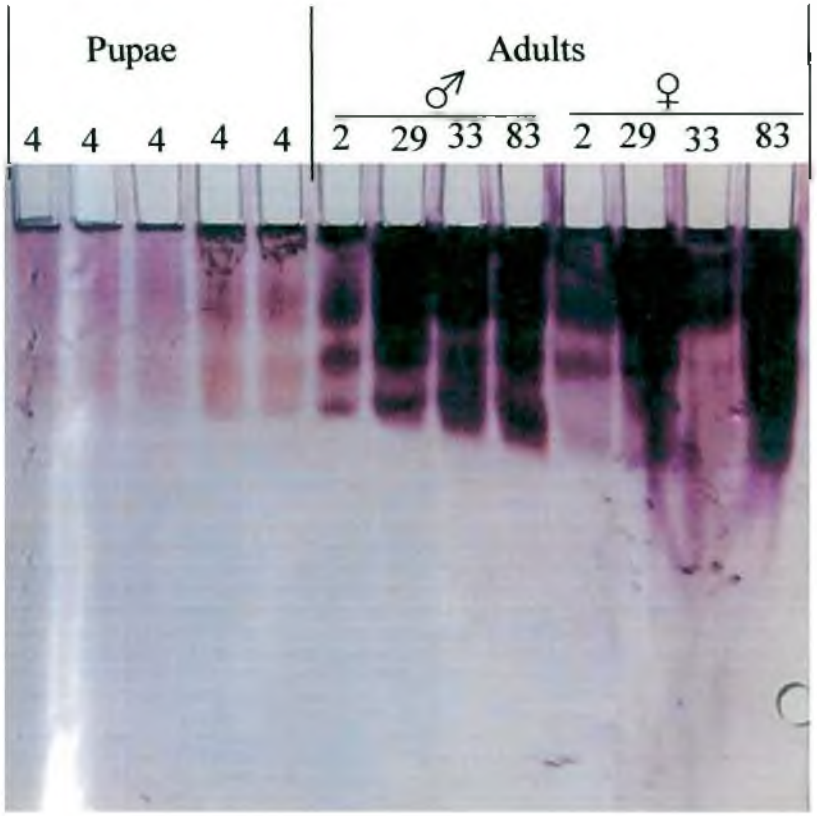
Fig. 1. Diagrammatic representation of seven esterase bands which are controlled by two esterase isozyme loci on the basis of their relative mobility (RM) values.

Table. 5. The proportion of EST-1 and EST-2 phenotypes in 56 individual of third instar larvae.

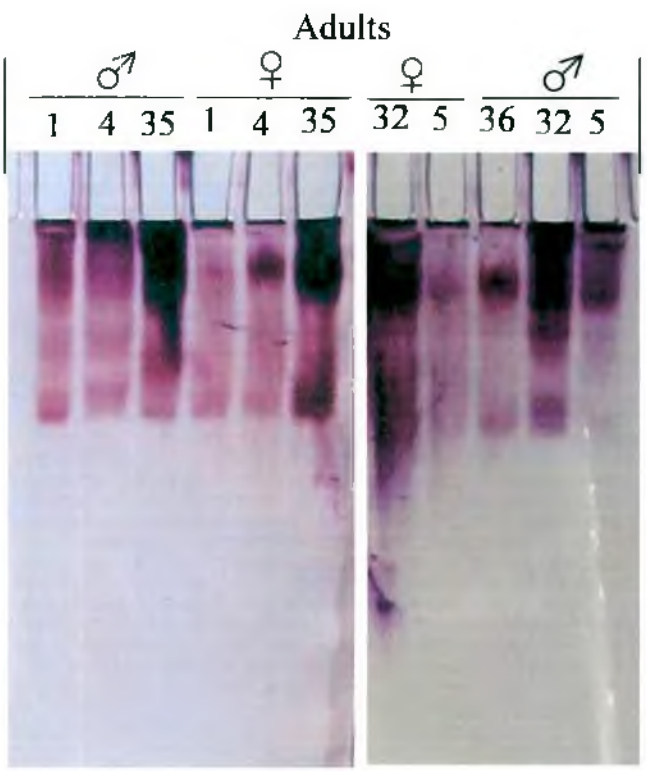
EST-1			EST-2		
Phenotypes	**Observed Nos.**	**Proportion**	**Phenotypes**	**Observed Nos.**	**Proportion**
Est-$1^{0.46}$	13	0.2321	Est-$2^{0.17}$	0	0
Est-$1^{0.46}$/Est-$1^{0.58}$	13	0.2321	Est-$2^{0.17}$/ Est-$2^{0.27}$	13	0.2321
Est-$1^{0.46}$/Est-$1^{0.87}$	0	0	Est-$2^{0.17}$/ Est-$2^{0.37}$	43	0.7679
Est-$1^{0.46}$/Est-$1^{1.00}$	30	0.5357		N=56	
	N= 56				

Table. 6. Allele frequencies (%) of gene at the EST-1 and EST-2 loci.

EST-1				EST-2			
Allele	**RM**	**Count**	**Frequency**	**Allele**	**RM**	**Count**	**Frequency**
Est-$1^{0.46}$	0.46	69	61.61	Est-$2^{0.17}$	0.17	56	50
Est-$1^{0.58}$	0.58	13	11.61	Est-$2^{0.27}$	0.27	13	11.61
Est-$1^{0.87}$	0.87	0	0	Est-$2^{0.37}$	0.37	43	38.39
Est-$1^{1.00}$	1.00	30	26.79	Total		112	100.00
Total		112	100.00				

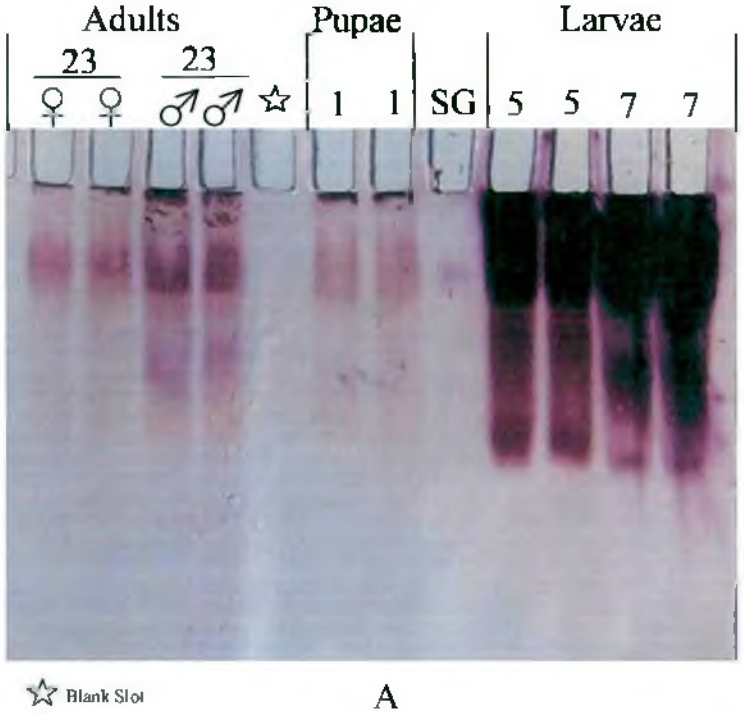


A

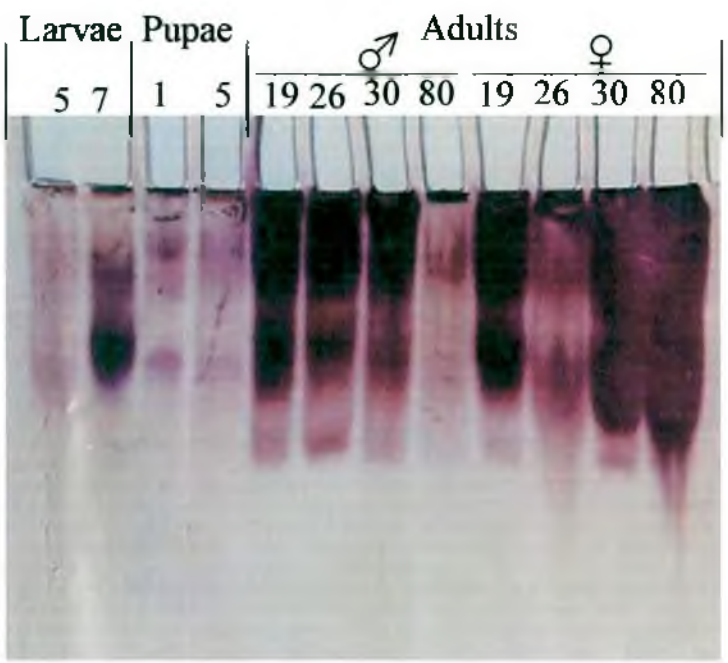


B C

Plate. 5. Electrophoretic banding patterns of esterase isozymes during the developmental stages (pupae and adults) of *B. cucurbitae*. Numbers above the slots indicate the age (days).

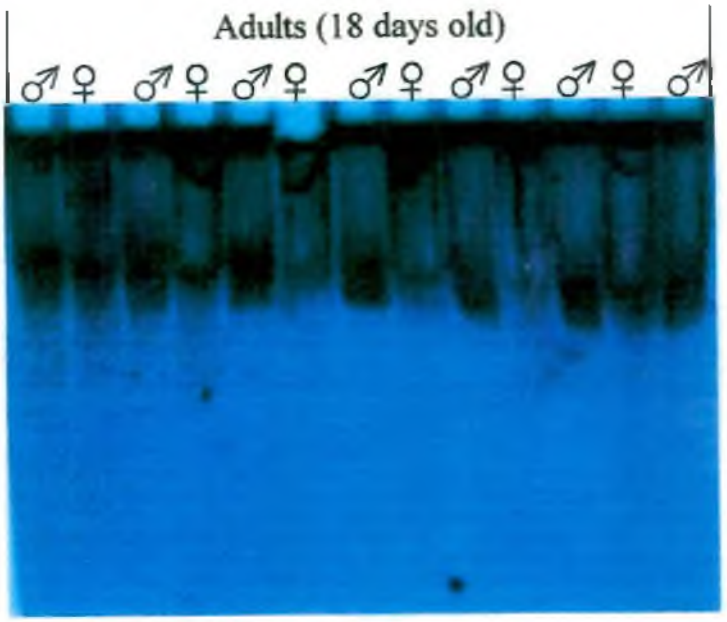


A

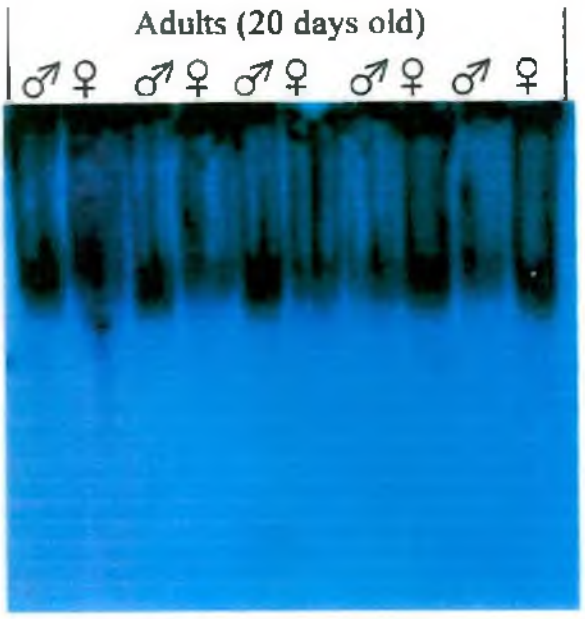


B

Plate. 6. Electrophoretic banding patterns of esterase isozymes during the developmental stages (larvae, pupae and adults) of *B. cucurbitae*. Numbers above the slots indicate the age (days) and SG means sweet gourd.



A



B

Plate. 7. Electrophoretic banding patterns of esterase isozymes in the adults (18 and 20 days old) of *B. cucurbitae*.

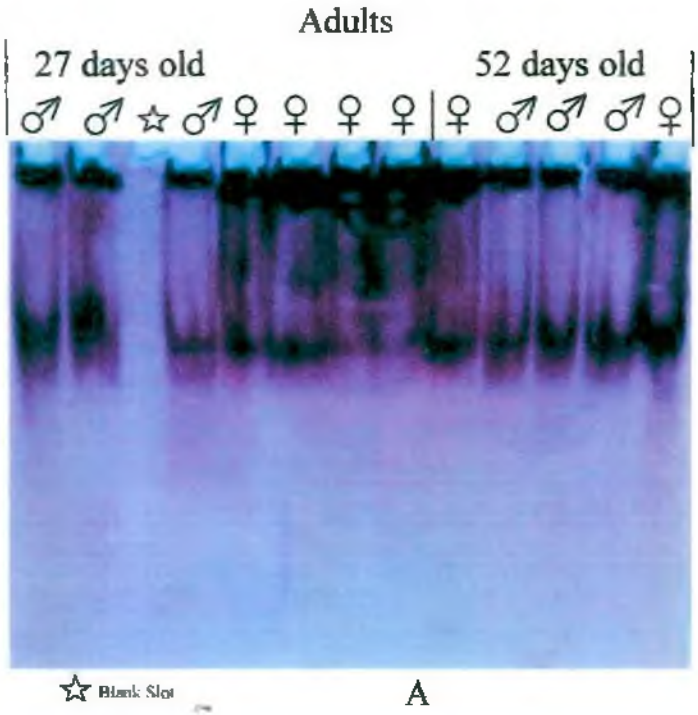
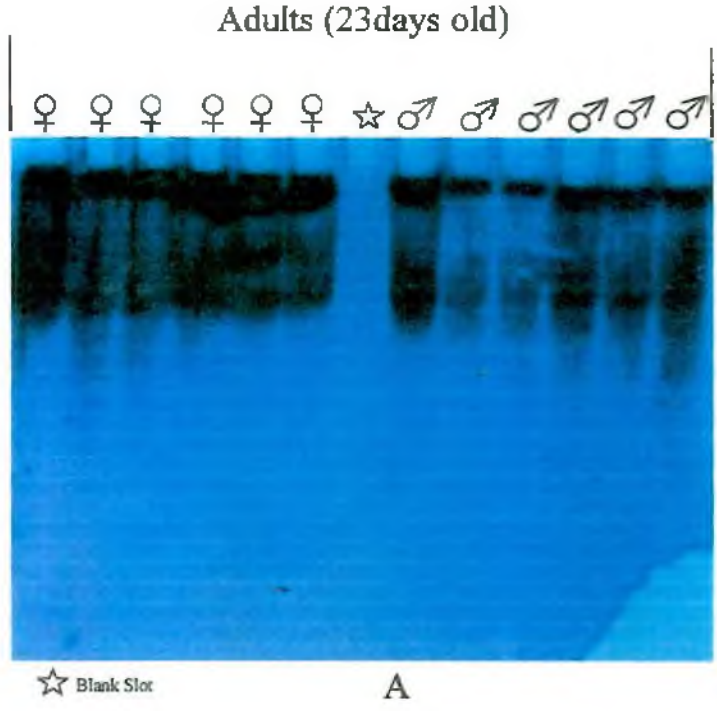


Plate. 9. Electrophoretic banding patterns of esterase isozymes in the adults (17, 27, 43 and 52 days old) of *B. cucurbitae*.



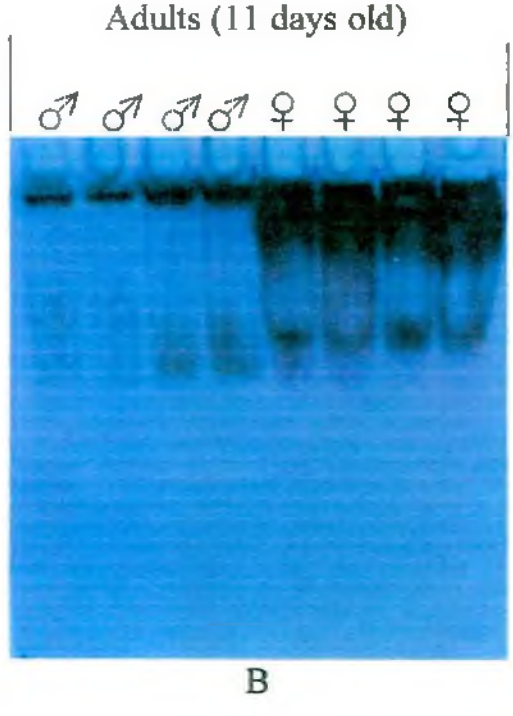


Plate. 10. Electrophoretic banding patterns of esterase isozymes in the adults (11 and 23 days old) of *B. cucurbitae*..

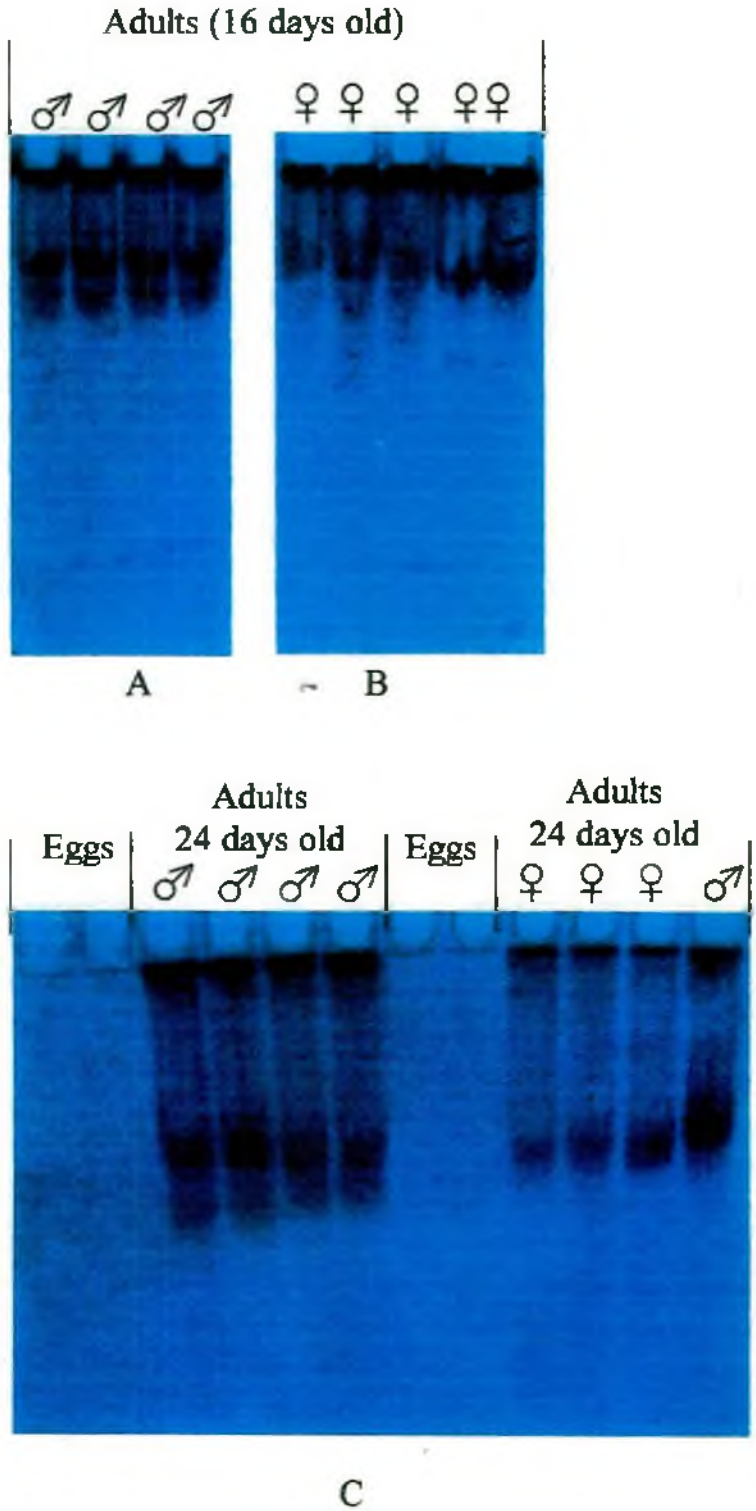


Plate. 11. Electrophoretic banding patterns of esterase isozymes in the eggs and adults (16 and 24 days old) of *B. cucurbitae*.

Table. 7. Esterase isozymes pattern* at the different life stages of *B. cucurbitae* (+ = present and - = absent).

Life Stages	Ages (Days)	Relative Mobility (RM) Values						
		EST-1				EST-2		
		1.00	0.87	0.58	0.46	0.37	0.27	0.17
Egg		-	-	-	-	-	-	-
Larva	1	-	-	-	-	-	-	-
	5	-	-	+	-	+	-	+
	6	-	-	-	+	-	+	+
	7	-	-	+	-	+	+	+
Pupa	1	-	-	-	-	+	+	+
	4	-	-	-	-	+	+	+
	5	-	-	-	-	+	-	+
Adult	1 (male)	-	-	-	+	-	+	+
	1 (female)	-	-	-	+	+	-	+
	2 (male)	-	-	-	-	+	+	+
	2 (female)	-	-	-	-	+	+	+
	4 (male)	-	-	-	-	+	+	+
	4 (female)	-	-	-	-	+	+	+
	5 (male)	-	-	-	-	-	+	+
	5 (female)	-	-	-	-	+	-	+
Sweet gourd		-	-	-	-	-	+	-

*scored from stained gel

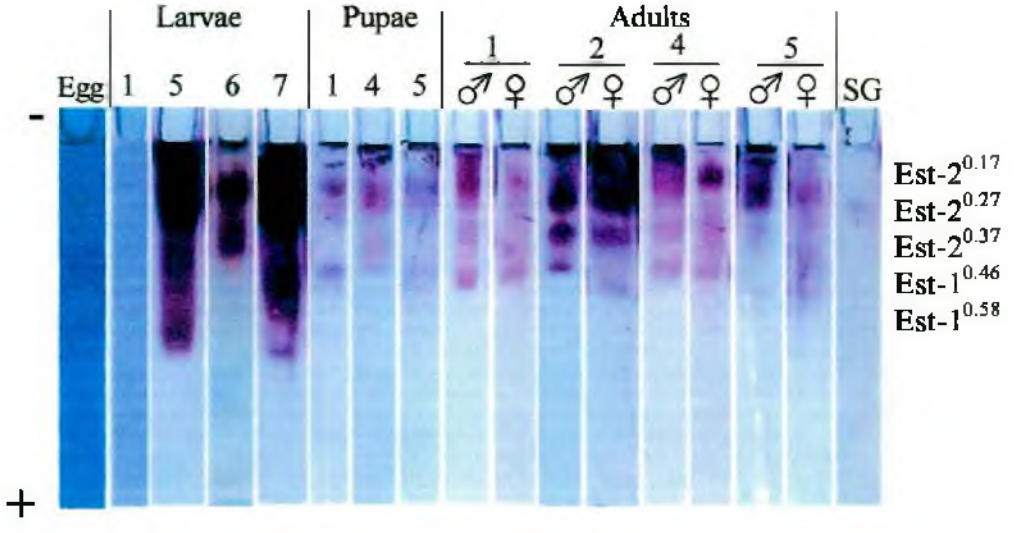


Plate. 12. Developmental profile of esterase isozymes in the melon fly, *B. cucurbitae*. Numbers above the slots indicate the age (days) of larvae, pupae and adults; and SG means sweet gourd.

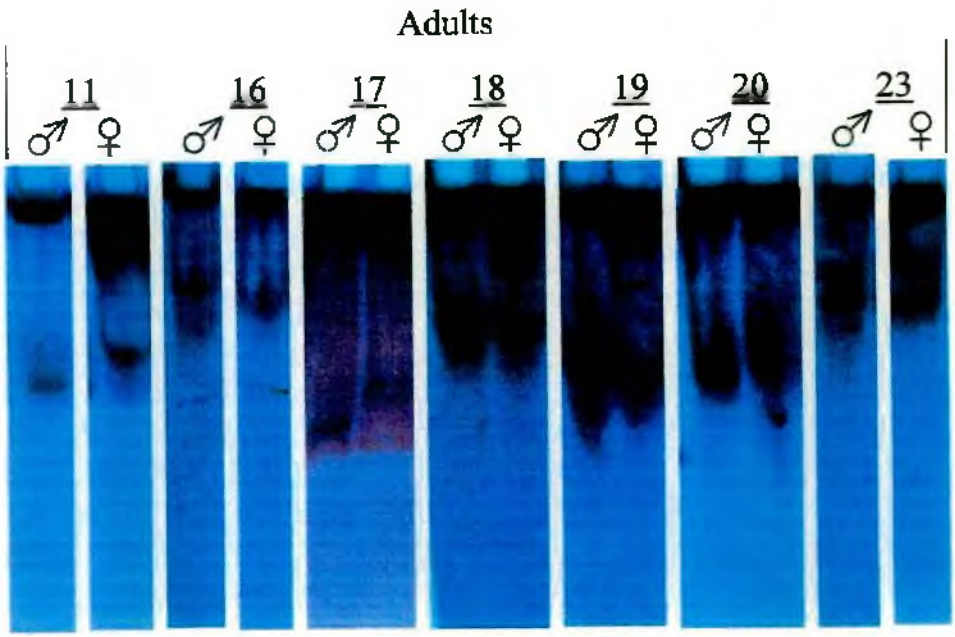


Plate. 13. Electrophoretic banding patterns of esterase isozymes in the adults (11, 16- 20 and 23 days old) of *B. cucurbitae*. Numbers above the slots indicate the age (days).

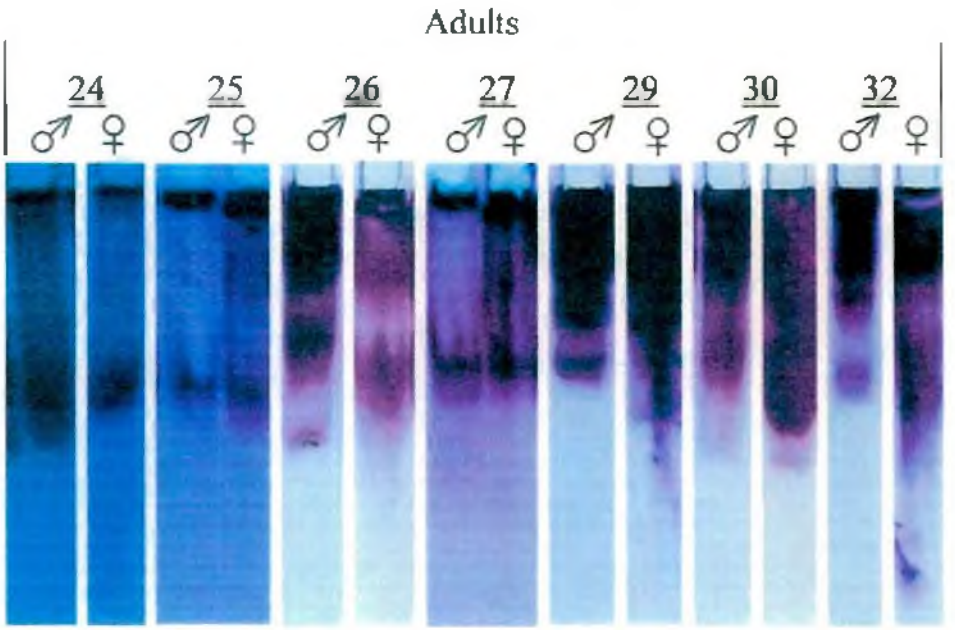


Plate. 14. Electrophoretic banding patterns of esterase isozymes in the adults (24- 27, 29, 30 and 32 days old) of *B. cucurbitae*. Numbers above the slots indicate the age (days).

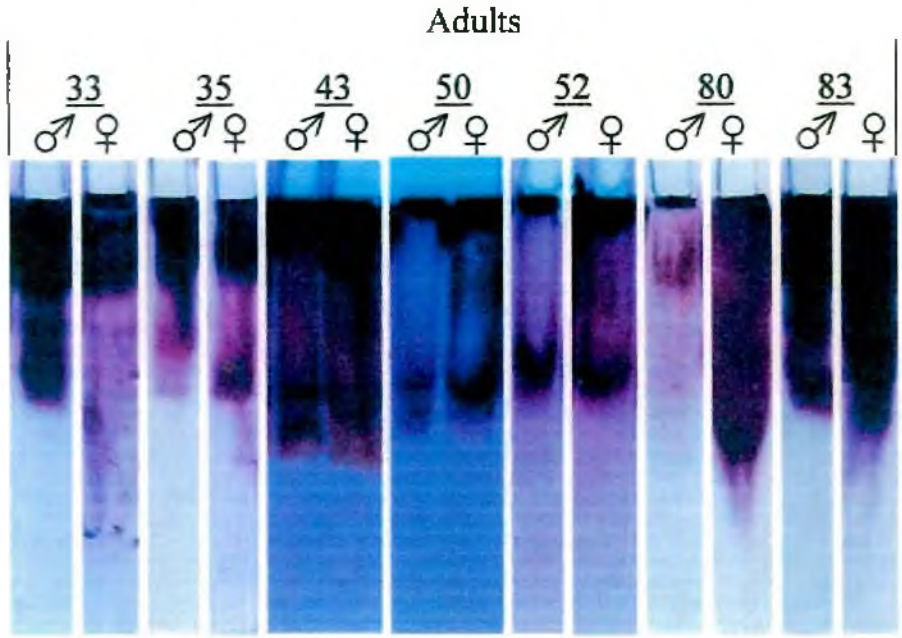


Plate. 15. Electrophoretic banding patterns of esterase isozymes in the adults (33, 35, 43, 50, 52, 80 and 83 days old) of *B. cucurbitae*. Numbers above the slots indicate the age (days).

4.2 POLYTENE CHROMOSOMES

4.2.1 SALIVARY GLAND

The larval salivary glands (**Plate 16**) are a pair of club shaped bodies attached to the pharynx. Each gland is made up of about 100 cells in dipteran insects (Hickman, 1970). With the aid of phase contrast microscope, the salivary gland cells appear as hexagonal honeycomb structure with large nuclei. Salivary tissue grows by an increase in cell number (Hickman, 1970). When the glands are stained with lacto-acetoorcein, the chromosomes can be seen more or less coiled up within the cells. When the cell membrane is disintegrated, the chromosomes are scattered out and can be easily studied.

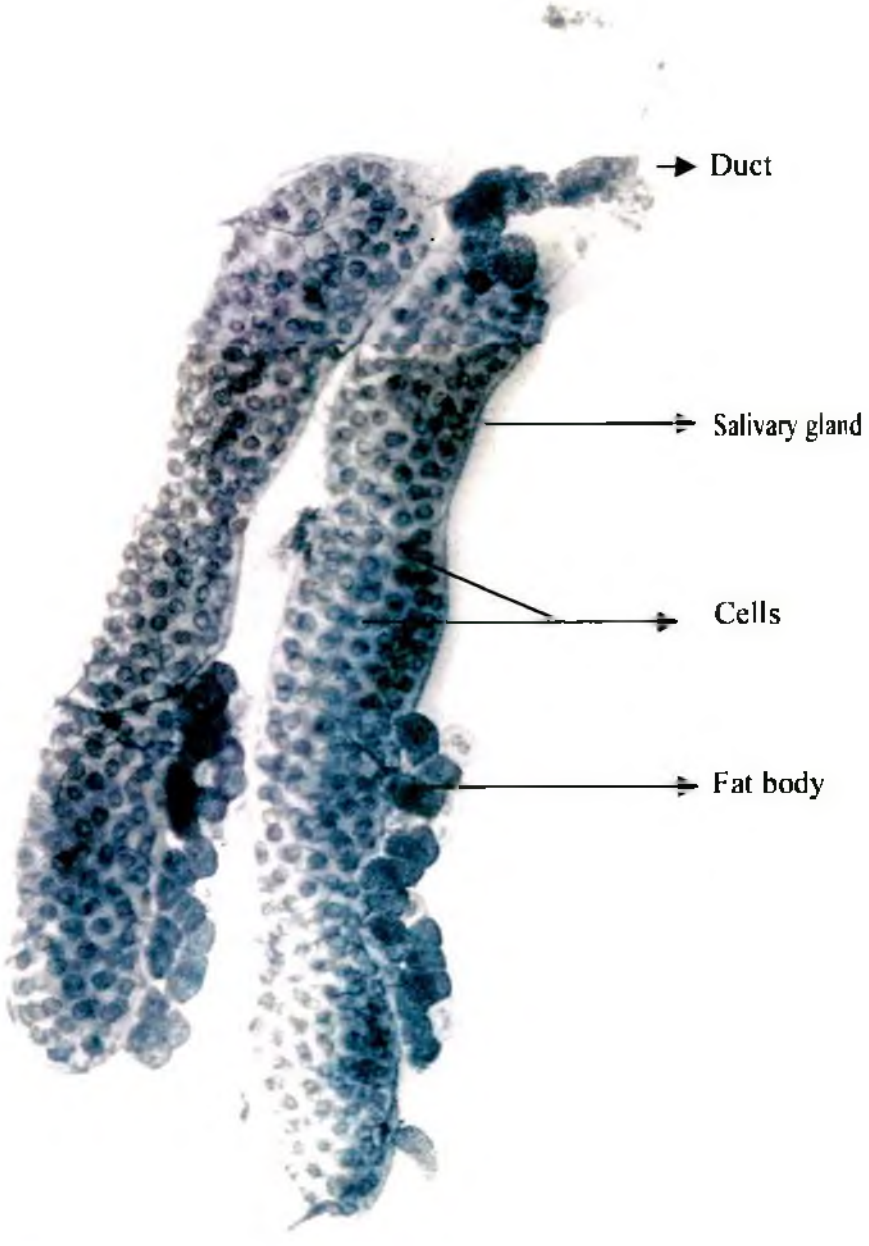


Plate. 16. Larval salivary glands of the melon fly, *Bactrocera cucurbitae*. 20X

4.2.2 BANDING PATTERN OF POLYTENE CHROMOSOMES

4.2.2.1 Photographic Maps of Polytene Chromosomes in 2nd Instar Larvae

The salivary gland polytene chromosomes of 2nd instar larvae are relatively more difficult as a cytological material. This is due to the fact that the chromosomes are smaller and the presence of extensive fragmentation, which occurs within, and between the chromosomes. Moreover, some chromosomal regions show very poorly defined banding pattern. Although chromosomes in 2nd instar larvae may be large and clearly banded, attempts to separate and spread them were usually unsuccessful because of the fragmentation of chromosomes at the weak points (sections 2, 19, 63, 76, 81, 91, 97 and 98), which usually break during slide preparation. Brief accounts of the banding patterns are as follows:

Sections 1-22 (Plate 17 and Fig. 2): Dark bands characterize the left end of the chromosome in 4A, 6 and 14A. Atsection 9 and 14B, each bears one pair faint bands. On the other hand, 15 comprises one pair moderately dark bands. The expanded regions in sections 3, 16 and 20 are not typical puff, rather section 16 bears a dotted band. The tip (section 1) has faintly stained two bands. Weak points are found in 2 and 19.

Sections 23-42 (Plates 17, 18 and Figs. 2, 3): Deeply stained bands occur in sections 30, 31 and 41. Dotted bands characterize 33 and 40 sections: 39 and 42 comprise two moderately developed puff regions. Breakage occurs at 26.

Sections 43-61 (Plates 18, 19 and Figs. 3, 4): This portion is characterized by a series of dark bands in 52, 55, 56 and 57. Sections 48 and 50 have similar single band. There is a poorly developed puff region in section 58.

Sections 62-81 (Plates 19, 20 and Figs. 4, 5): Lightly stained two pairs of bands present in section 68. Sections 72, 73, 74 and 79 possess single band. One pair lightly stained bands occurs at 77 and 78. There is a prominent puff in section 80 together with a dark band immediately proximal to it. Weak points are found in 63, 76 and 81 sections.

Sections 82-100 (Plate 20 and Fig. 5): Section 83 possesses three clear bands. A series of lightly stained and deeply stained bands are found in the sections 87 and 95. Section 100 appears only as a well-developed puff in the total polytene chromosome complement. Three weak points are found in 91, 97 and 98.

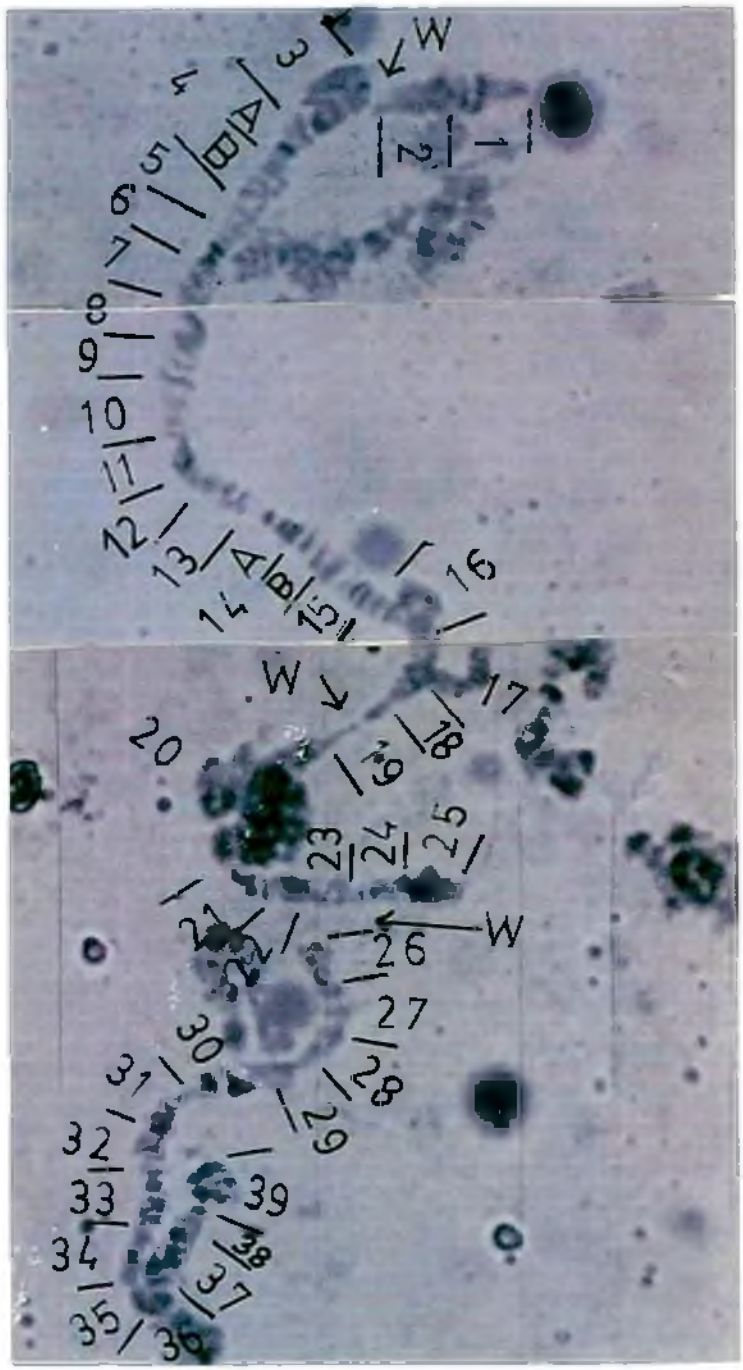


Plate. 17. Salivary gland polytene chromosome maps of 2nd instar larvae of *B. cucurbitae*, sections 1- 39. Several weak points (W) where chromosome fragmentation is evident are marked by arrows.

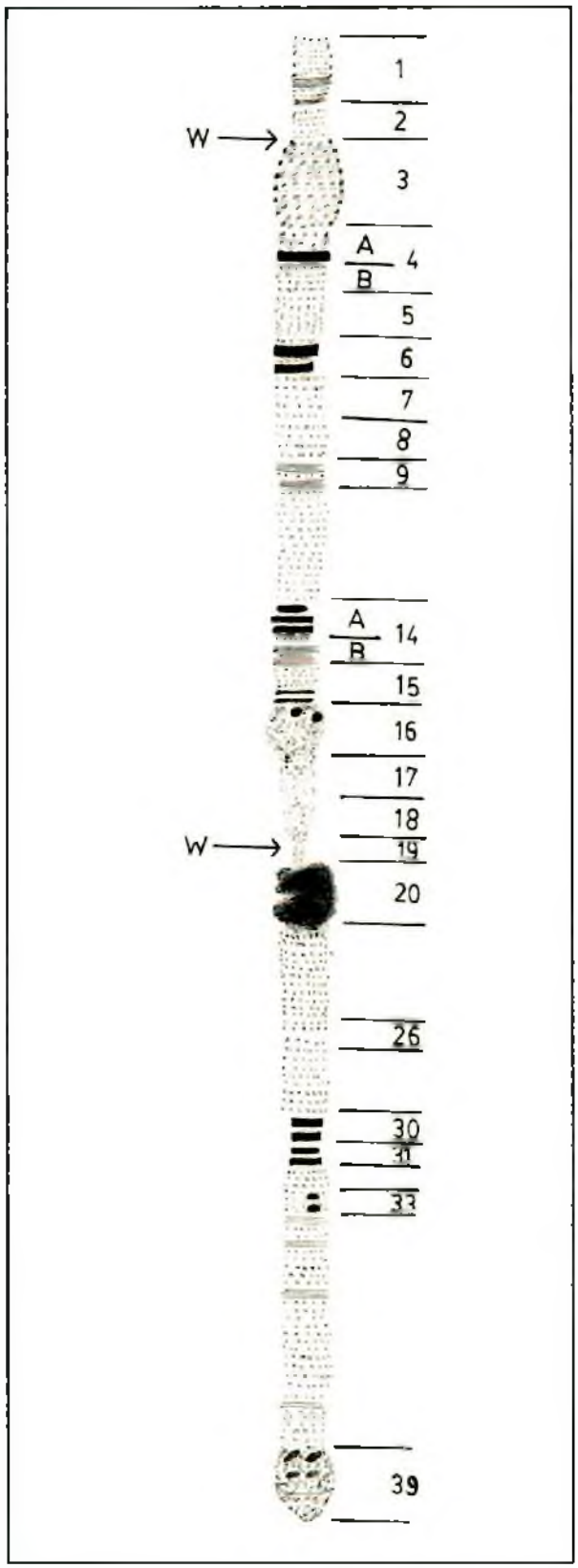


Fig. 2. Diagrammatic representation of the salivary gland polytene chromosome maps of 2nd instar larvae of *B. cucurbitae*, sections 1- 39. W = Weak point.

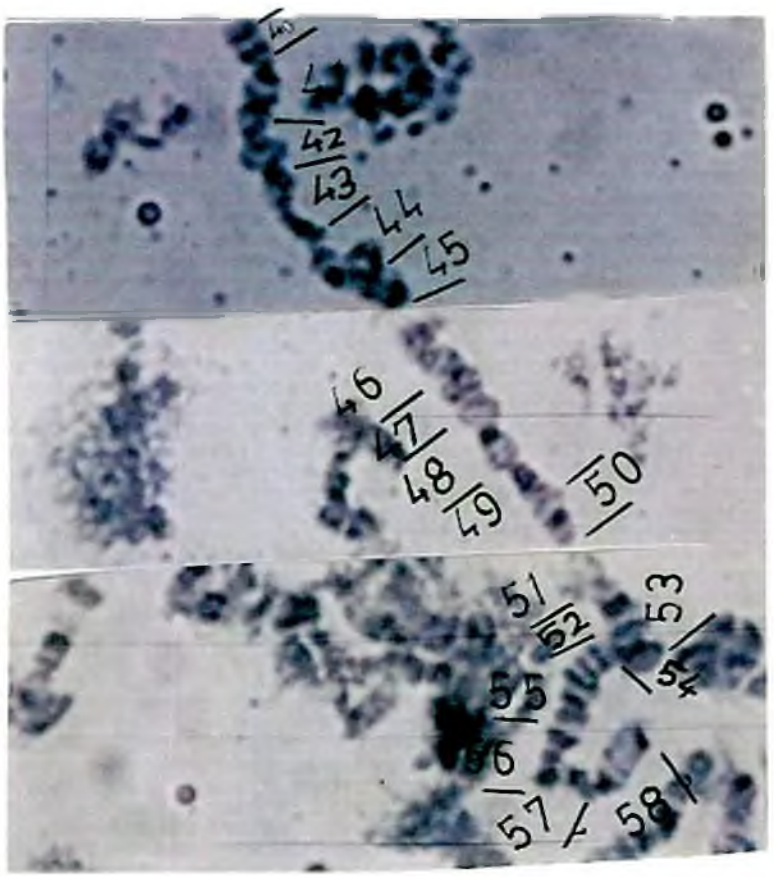


Plate. 18. Salivary gland polytene chromosome maps of 2nd instar larvae of *B. cucurbitae*, sections 40- 58.

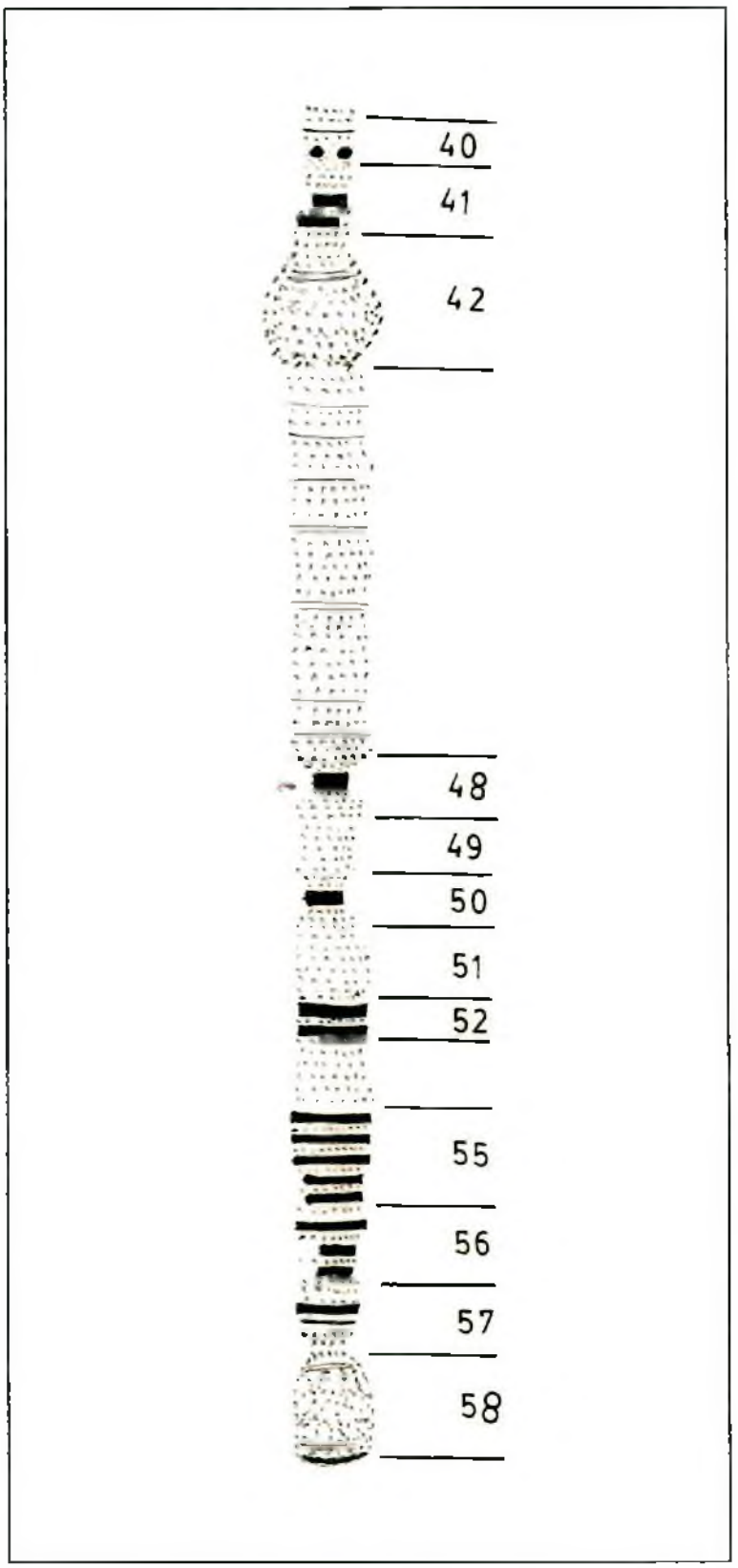


Fig. 3. Diagrammatic representation of the salivary gland polytene chromosome maps of 2nd instar larvae of *B. cucurbitae*, sections 40- 58.

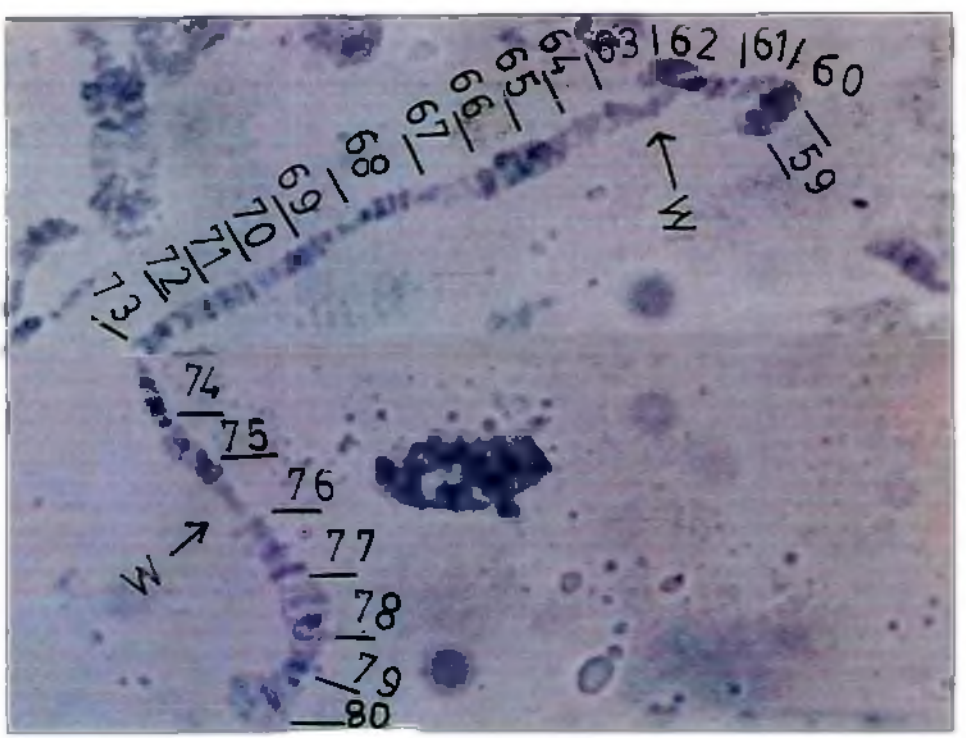


Plate. 19. Salivary gland polytene chromosome maps of 2nd instar larvae of *B. cucurbitae*, sections 59- 80. Several weak points (W) where chromosome fragmentation is evident are marked by arrows.

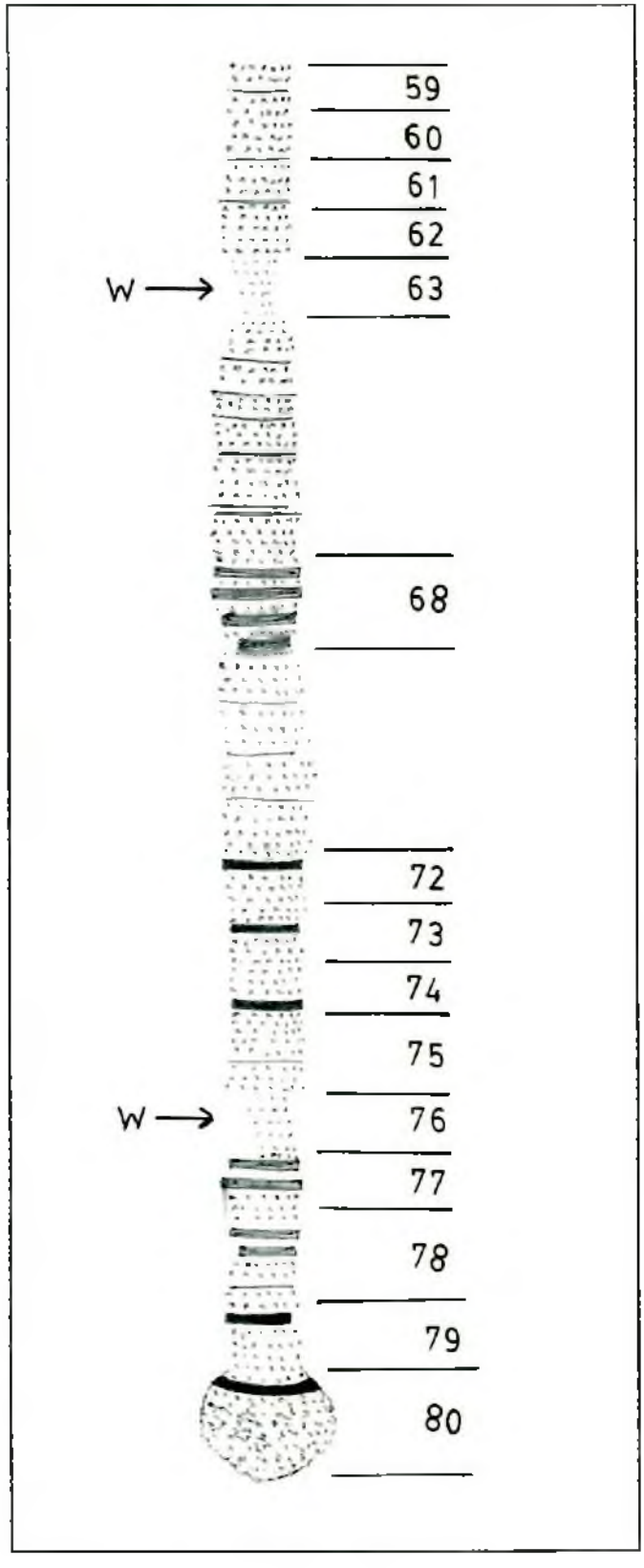


Fig. 4. Diagrammatic representation of the salivary gland polytene chromosome maps of 2nd instar larvae of *B. cucurbitae*, sections 59- 80. W = Weak point.

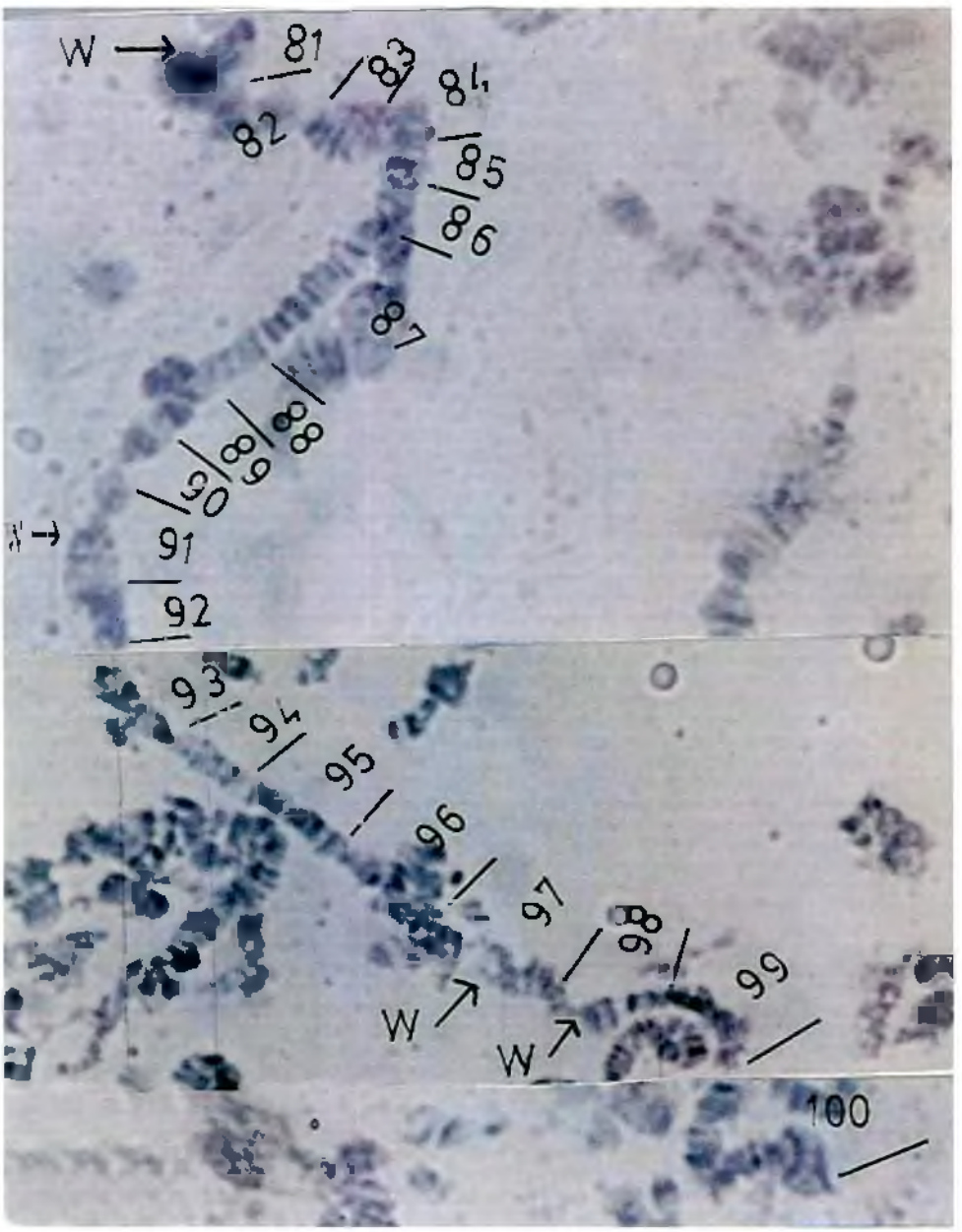


Plate. 20. Salivary gland polytene chromosome maps of 2nd instar larvae of *B. cucurbitae*, sections 81- 100. Several weak points (W) where chromosome fragmentation is evident are marked by arrows.

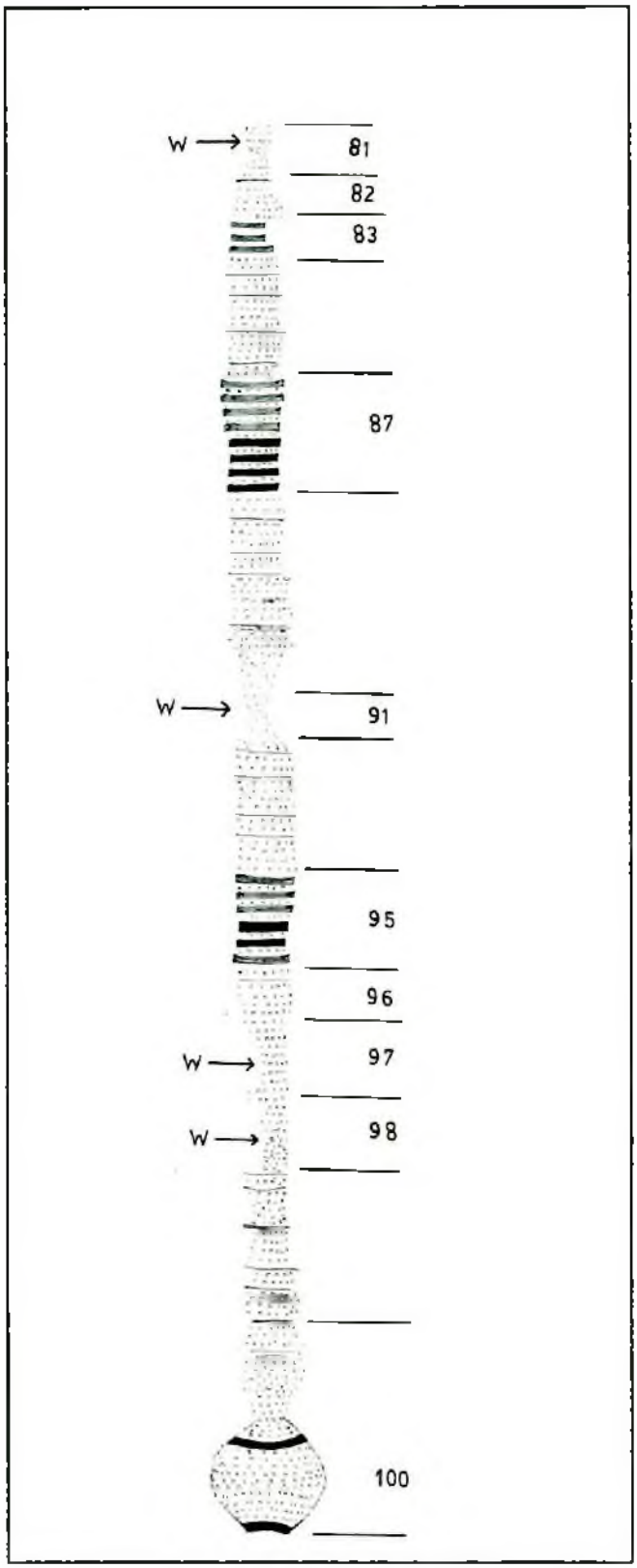


Fig. 5. Diagrammatic representation of the salivary gland polytene chromosome maps of 2nd instar larvae of *B. cucurbitae*, sections 81- 100. W = Weak point.

4.2.2.2 Polytene Chromosomes in 1st Instar Larvae

The band formation is very weak in the polytene genome of 1st instar larvae (**Plate 21**). At this stage the formation of salivary gland cells as well as polytene chromosomes are at their early stages of maturity. So that, any distinctive features or characteristics could not be precisely localized in the polytene chromosomes.

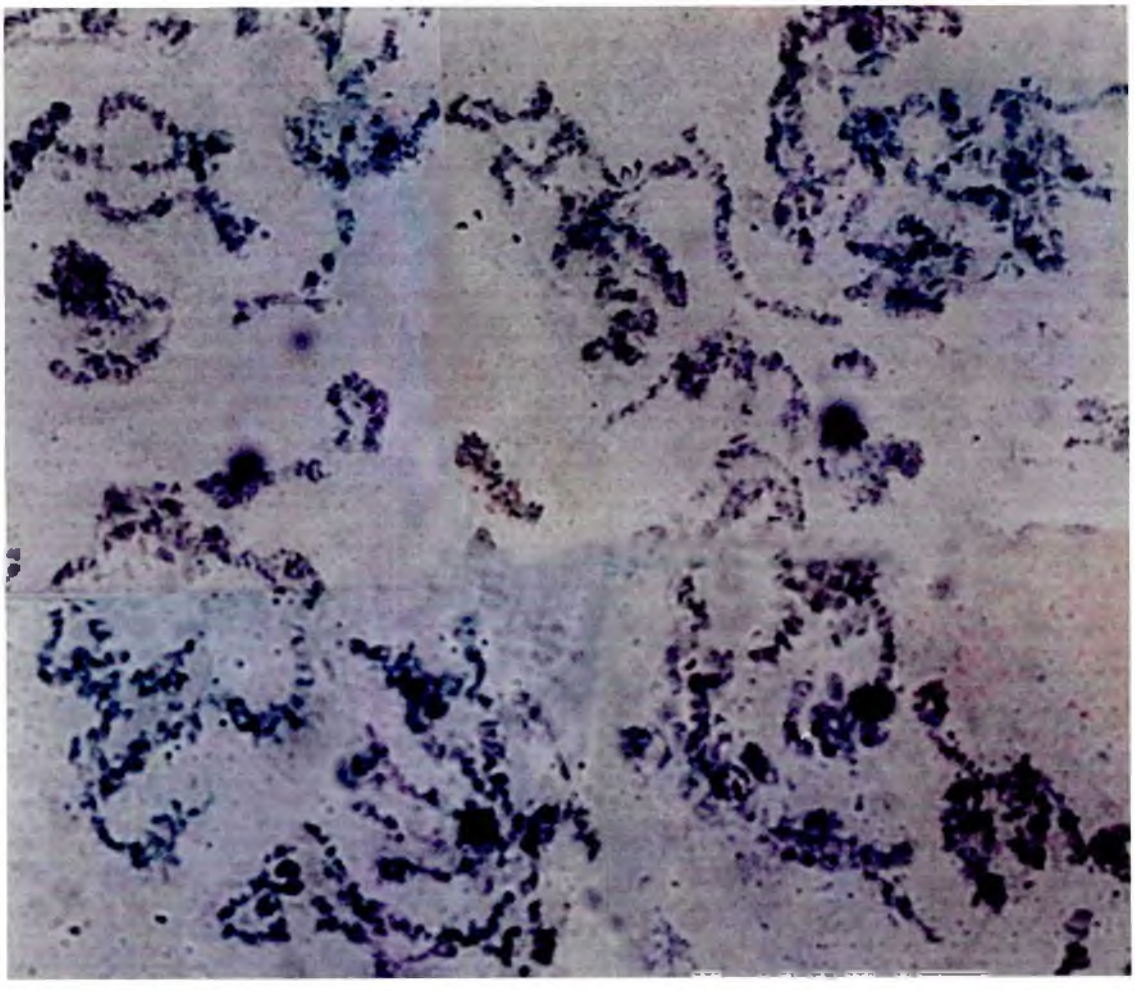


Plate. 21. Salivary gland polytene chromosomes of 1st instar larvae of *B. cucurbitae*.

Chapter-5

Discussion

DISCUSSION

The present research requires a multidisciplinary discussion in respective fields. But in some cases, it is difficult to assess the test results for the lack of baseline data. The discussions are as follows in detailed:

5.1 ESTERASE ISOZYME VARIATIONS

The esterase polymorphism detected in the different life stages of *B. cucurbitae*, about seven esterase bands for protein electrophoresis (**Fig. 1**). These bands resulting for α and β napthyl acetate showed little variation of mobility. These slight variations possibly occurred for two factors. One is, all the individual samples were not tested on the same and so plate to plate variation may account for the differences; and the other is buffer solution from experiment to experiment. The individual bands showed variation in the relative mobility was, band of RM 1.0 ranging from 0.96 to 1.04, band of RM 0.87 ranging from 0.83 to 0.91, band of RM 0.58 ranging from 0.54 to 0.68, band of RM 0.46 ranging from 0.42 to 0.50, band of RM 0.37 ranging from 0.33 to 0.41, band of RM 0.27 ranging from 0.23 to 0.31, band of RM 0.17 ranging from 0.13 to 0.21 for these individuals samples. So, their mean relative mobility was considered as the standard relative mobility.

All the seven bands were not found in one individual. Maximum six different bands and minimum one band was found in an individual sample. Majority of the individuals contained 3 to 4 different esterase bands. This large number of variation of individual bands indicated that the variability for esterase was high in this population.

Individual samples were stained for esterase activity by α and β napthyl acetate using gel electrophoresis. Alpha-napthyl acetate is responsible for black colour and β napthyl acetate is responsible for red colour (Steiner and Joslyn, 1979). But no noticeable differences were found in the present study, all the bands stained the same red colour. Trebatoski and Craig (1969) and Townson (1971) revealed different esterase locus of *Ae. aegypti* according to their relative mobility. Although all seven bands stained the same red colour, some of the bands stained more intensely than others.

The seven different bands observed during the study, present in 2 different esterase isozyme loci was assumed on the basis of the relative mobility, staining intensity of alleles and previous literature

survey. These are controlled by two different esterase loci with seven alleles.

Polyacrylamide gel electrophoresis of the different life stages of *B. cucurbitae* revealed two esterases, EST-1 and EST-2, which were designated from the highest to lowest relative mobilities, that is, from the lowest molecular weight to highest molecular weight. In *Culex* spp., resistance-associated enzymes have much lower relative mobilities than those of either the larvae or the adults of *Anopheles subpictus* and are much larger enzymes with high molecular weight (Hemingway *et al.*, 1987).

In the larvae, the quantity of the enzymes appeared to be higher than that of the eggs, pupae and adults as visualized in the gels (**Plates 4 and 12**). Among the pupae and adults it seems that esterase activity is lower than larvae. There are numerous examples of differences in the level of resistance at the larval and adult stages, as in *Culex quinquefasciatus* with over 1000-fold temephos resistance and 100-fold malathion resistance in the larvae but much lower levels of resistance in the adults and *A. stephensi* with 24-fold malathion resistance in the adults and 6-fold resistance in the larvae (Georghiou *et al.*, 1980; Hemingway and Georghiou, 1983). In all these cases, the

highest level of resistance is reflected in the life stage on which the major selection pressure has been occurred (Hemingway *et al.*, 1987).

In *B. cucurbitae*, the alleles detected at EST-1 (Est-$1^{0.46}$, Est-$1^{0.58}$, Est-$1^{0.87}$ and Est-$1^{1.00}$) and EST-2 (Est-$2^{0.17}$, Est-$2^{0.27}$ and Est-$2^{0.37}$), are low in numbers. Studies by Pasteur & Sinegre (1975) and de Stordeur (1976) of the esterase-2 locus in a southern France population found seven and nine alleles respectively by using starch gel electrophoresis. At the protein level, 18 and 16 alleles were found for esterase-3 and 2 respectively in one French population (n = 74), and 16 and 14 in English one (n = 50), and at the DNA level, 24 alleles at the esterase-2 locus were detected in a sample of 72 mosquitoes from one population. These large number of alleles found by Raymond *et al.* (1996) which can be explained by the fact that they studied the susceptible populations. In susceptible population of Raymond *et al.* (1996), no mosquito displayed an esterase band with high activity. It indicates that the low activity esterase bands are less fit and their frequencies of the population have been reduced or became zero (0) by the selection pressure given by frequent use of insecticides for long period of time. In susceptible mosquitoes, Est-2 and Est-3 have different electrophoretic mobilities, corresponding to many different alleles (Georghiou *et al.*, 1980).

Since other allozymes of these proteins exist, the finding of such a narrow distribution of allozymes would seem difficult to explain on any basis other than selection (Strickberger, 1996). One of the examples of such condition is Milkman (1973) found that, in *E. coli* clones for each of five different enzymes, one particular electrophoretic band was frequent in almost all samples. In case of *B. cucurbitae*, Est-$1^{0.46}$ and Est-$2^{0.17}$ were also found in all third instar (6 days old) larvae (**Plates 2, 3 and 4**).

The overproduced and heavy bands of EST-1 and EST-2 may be the result of gene amplification at these loci. Co-amplification of two esterases is the most common organophosphate insecticide resistance mechanism (Hemingway *et al.*, 1998; Mouches *et al.*, 1986; Raymond *et al.*, 1998). Typically, the amplification (amplified gene) is inherited as a single dominant gene (Takada, 1978; Blackman *et al.*, 1996; Ferrari & Georghiou, 1991). Among the overproduced bands, Est-$1^{0.46}$ seems to be more heavy than Est-$2^{0.17}$ (**Plate 4**). This is also reported by Hemingway *et al.* (1998) who stated that the amplified esterases are differentially regulated, with three times more Est-$1^{0.46}$ (Est-β2′ in their paper) being produced than Est-$2^{0.17}$ (Est-α 2′ in their paper).

According to Raymond *et al.* (1996), presence of more than two bands where all the bands had the same relative mobility as one of the bands detected in the other individual, suggests that they are not artifactual. According to previous Mendelian inheritance studies on these esterase loci (de Stordeur, 1976), each band corresponds to one allele. Normally in diploid individuals, more than two alleles can not be present at one locus at a time. The presence of melon fly displaying three or four esterase bands at one locus indicates the presence of gene duplication within the *Bactrocera cucurbitae* population (typically **Plate 4**). It is now clear that such gene duplications are not unusual and can easily arise from an unequal crossing over event that produces a recombinant product possessing increased chromosome materials (Strickberger, 1996). Esterase gene duplication has been reported in several insect species (Brady & Richmond, 1992; East *et al.*, 1990), but in the mosquito *Culex pipiens* it was first reported by Raymond *et al.* (1996).

Zouros and Krimbas (1970) analysed a three bands family in *Dacus oleae* and concluded that the only possible explanation is a duplication. Since individuals bearing duplication have even more enzymes than individuals bearing only two active gene copies, it should be expected that the duplications would be selected for. This is in fact observed during the present study.

Alternately, three or four bands can be explained as a result of heterozygosity in which additional intermediate bands appear. This condition has been found by many workers in many species (Johnson & Denniston, 1964; Riva and Robinson, 1986 and Gasperi *et al.*, 1992). If this is true for *Bactrocera cucurbitae*, although less probable, heterozygote advantage or overdominance seems to exist in the population. In this condition, the phenotypic expression of a heterozygote is more extreme than that of either homozygote (Strickberger, 1996) and heterozygote are selected for.

No null individuals have been found at EST-1 and EST-2 loci. As exhaustive sampling is practically impossible, their absence can not be firmly established. It is still possible that they are present in heterozygous condition in the studied samples.

Absence of null individuals can be explained alternately. Possibly null individuals are not found because null alleles do not produce active enzymes that detoxify the insecticides. Since, they can not detoxify insecticides, null allele bearers become less fit against insecticides. There is rational cause of their low frequency in the

population with insecticidal selection pressure. Thus, in a resistant population, absence of null alleles can be justified.

5.2 POLYTENE CHROMOSOMES

To increase the depth of knowledge in cytogenetic analysis, attempts were made for the first time to construct a photographic maps of the polytene genome of 2nd instar larvae in *B. cucurbitae*.

Previous reports on the polytene map of *Dacus* (*Bactrocera*) *cucurbitae* (Singh and Gupta, 1984) have been described in the larval salivary glands. However, these were drawings of polytene maps and do not facilitate cytogenetic analysis and are also difficult for further analyses. A detailed photoghraphic maps and also sketches of 3rd instar larval salivary gland polytene chromosomes were presented by Shahjahan and Yesmin (2002). Generally polytene nuclei were characterized by many constrictions and weak points in the chromosomes (Bedo, 1986). Most of the cytogenetists, working for several important pest species such as *Ceratitis capitata*, *Lucilia cuprina*, *Dacus oleae*, and *Bactrocera tryoni*, reported that the salivary gland chromosomes were very difficult to spread because of extensive ectopic pairing. In most cases where the chromosomes spread, they were broken and it was difficult to distinguish each

chromosome as a separate entity. We also observed such difficulties for *B. cucurbitae*.

In 3rd instar larvae of this species, Shahjahan *et al.* (2002) and Shahjahan & Yesmin (2002) reported that each polytene nucleus had five pairs of long chromosomes (10 polytene arms, I-V). These are identifiable by their diagnostic landmarks as well as their characteristic tips. There are no differences between preparations from male and female, indicating that the sex chromosomes are not polytenized. This is also the case in *C. capitata* and in *L. cuprina* (Bedo, 1980 & 1987; Foster *et al.*, 1980; Zacharopoulou, 1987 & 1990). In this work, the total polytene chromosome complement of 2nd instar larvae was also divided into 100 sections, but it is not possible to confidently assign chromosome numbers; because no distinct centromeric position could be ascertained as found in 3rd instar larvae (Shahjahan *et al.*, 2002 and Shahjahan & Yesmin, 2002).

In the present work, within the polytene nuclei of *B. cucurbitae*, no banded sex chromosomes were also identified. These chromosomes were represented by a heterochromatic structure in 3rd instar larvae (Shahjahan & Yesmin, 2002). Such type of heterochromatic structure was absent at this stage. That structure may be broken during slide preparation. The presence of sex

chromosomes in the form of heterochromatic mass were also identified in 3rd instar polytene tissues of *C. capitata*, *L. cuprina*, and *B. oleae* (Childress, 1969; Bedo, 1982 & 1987; Bedo and Zacharopoulou, 1988; Zambetaki *et al.*, 1995). Banded sex chromosomes have not been observed in polytene nuclei of several other Diptera, e.g., *Calliphora* and *Sarcophaga* (Whitten, 1968). Any sex chromosomes or sex-linked segments could not be detected in *Simulium* spp. (Hadi *et al.*, 1995). However, further studies are necessary for this clarification.

The characteristic features of the banding sequences of the 1st and 2nd instar larvae have been summarized as follows:

(i) Band formation is very weak and indistinct in 1st instar larvae.

(ii) Banding pattern is moderately developed in 2nd instar larvae and the presence of a large number of weak points.

Such chromosome banding techniques may be allowed rapid advancement of our understanding of cytogenetic mechanisms and facilitate chromosome manipulation, and devising safe way control strategies in this important pest species.

Chapter-6

Summary

SUMMARY

Insect pests are increasing problem in agriculture and intervention is required to limit losses in crop production. Some insects can be the cause of trade embargoes arising from strict quarantine regulations, or can infest commodities, which require costly and hazardous chemical post-harvest treatments. Insect pests often hamper economic development and the well being of a growing human population. About 4.5 x 10^{6} ton of vegetables and 1.5 x 10^{6} ton of fruit are produced in Bangladesh annually. Usual loss of fruits and vegetables to insects ranges from 10-15%. In some cases, it causes a total loss of the crop (SYBB, 1999). Different eradication measures are constantly being explored to alleviate the problem but as yet relatively little research has been conducted on genetical control methods which involve various forms of chromosome analysis (i.e., basic banding pattern, translocation, inversion hybridity) and systems of different isoenzyme activity. An essential prerequisite in these types of work is a thorough understanding of the normal chromosome complement and how the various isozymes behave during their life stages. The present research works try to explain a brief outline of these respects.

Polyacrylamide gel electrophoresis (5%) of the different life stages of *B. cucurbitae* revealed two esterases, EST-1 and EST-2, which were designated from the highest to lowest relative mobilities (**Fig. 1**), that is, from the lowest molecular weight to highest molecular weight. There were four electromorphs in EST-1 (Est-$1^{0.46}$, Est-$1^{0.58}$, Est-$1^{0.87}$ and Est-$1^{1.00}$), had a high mobility and close to the anode and EST-2 had three allelomorphs (Est-$2^{0.17}$, Est-$2^{0.27}$ and Est-$2^{0.37}$), showed a low mobility and close to the cathode (**Plate 4 & 12**).

These bands resulting for α- and β-napthyl acetate showed little variation of mobility. This slight variation possibly occurred for two factors:

- All the individual samples were not tested on the same and so plate to plate variation may account for the differences.
- The other is buffer solution from the different experiments.

All the seven bands were not found in one individual. Maximum six different bands and minimum one band was found in an individual sample. Majority of the individuals contained 3 to 4 different esterase bands. This large number of variation of individual bands indicated that the variability for esterase was high in this population. In the larvae, the quantity of the enzymes appeared to be higher than that of the eggs, pupae and adults as visualized in the gels

(**Plate 4 & 12**). Among the pupae and adults it seems that esterase activity is lower than larvae. However, Est-$1^{0.46}$ and Est-$2^{0.17}$ were also found in all third instar (6 days old) larvae of *B. cucurbitae* (**Plate 2, 3 & 4**). In EST-1, the allele frequencies obtained have been presented in the **Table 6**. Thus, Est-$1^{0.46}$ was in the highest frequency 61.61%, in the population. Next highest frequency, 26.79%, was found for the Est-$1^{1.00}$ allele and 11.61% for Est-$1^{0.58}$ allele. Frequencies of the different alleles at EST-2 locus were calculated with the assumption that all the single band expressions were the result of homozygosity for the respective alleles. This provided the highest frequency for the Est-$2^{0.17}$ allele that was 50%. Next highest frequency, 38.39% was found for the Est-$2^{0.37}$ allele and 11.61% for Est-$2^{0.27}$ allele (**Table 6**).

The standard photographic maps of salivary glands polytene chromosomes were also observed in the different larval stages (1^{st} and 2^{nd} instar larvae) of *B. cucurbitae*. In 2^{nd} instar larvae, the whole polytene genome had been mapped by dividing it into 100 sections and the sub sections were lettered, depending on its characteristic features and landmarks (**Plates 17- 20 and Figs. 2- 5**). The banding pattern was moderately developed here. The left end of the chromosome is characterized by dark bands in 4A, 6 and 14A. Sections 9 and 14B, each bears one pair faint bands. The expanded regions in sections 3, 16 and

20 are not typical puff, rather section 16 bears a dotted band. Sections 39 and 42 comprise two moderately developed puff regions. There is a poorly developed puff region in section 58. One pair lightly stained bands occurs at 77 and 78. There is a prominent puff in section 80 together with a dark band immediately proximal to it. Section 83 possesses three clear bands. Section 100 appears only as a well-developed puff in the total polytene chromosome complement. In the case of 1st instar larvae, salivary gland cells were at early stages of maturity. So the banding pattern of polytene genome was not developed at this stage (**Plate 21**). This photographic polytene chromosome maps provide a means to pinpoint the precise location of a chromosome breakpoint or a rearrangement.

The significance of studying the polytene chromosome complement of *B. cucurbitae*, which causes serious damage to the fleshy fruits and vegetables, is evident. In this thesis, it is tried to determine the chromosomal characteristics of this species so that these data can be used as reference material for further cytogenetic analysis. With the success in finding good cytological material in *B. cucurbitae*, genetic studies can now proceed with excellent cytological support. This work has revealed the fact that this species has a readily workable cytological system of basic interest, which may provide interesting results in future.

It has been estimated that insect pests reduce the world's food supply by 25%. Insect pest management is therefore an important strategic component in raisi ng food production and ensuring food security, and in protecting forests, stored products and fibre crops. For many years insect control methods were based primarily on insectici des. The consumption of insecticides is increasing worldwide at about 5% each year. The present reliance on chemical pesticides to control insect pests can not be sustained in the future. Increasingly, there is pesticide resistance in target pests, as well as the emergence of new pests as a result of the elimination of their natural enemies by insecticides. Furthermore, public concerns have grown considerably as a result of widespread environmental pollution, contamination of groundwater, and the presence of pesticides residues in food and fibre. In view of this unsustainable reliance on chemical pesticides and public concerns, new and more environment-friendly techniques, approaches and strategies in the war against insect pests are called for. New legislation is restricting more and more the use of insecticides.

Pest control interventions today are increasingly being implemented within the concept of pest control by genetic

manipulation, with more attention being given to ecological and environmental concerns.

Pest control by genetic manipulatiuon results in more sustainable, environment-friendly and long-term pest control tactics/strategy. A study of polytene chromosome mapping and esterase isozyme analysis in *B. cucurbitae* is a useful tool to develop rapid advancement of our understanding of genetic manipulation.

Chapter-7

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