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Population genetics of Helicoverpa zea (Boddie) (Lepidoptera: Noctuidae): differentiation and quantification of overwintering and spring migratory populations in northern Mississippi

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POPULATION GENETICS OF *HELICOVERPA ZEA* (BODDIE) (LEPIDOPTERA: NOCTUIDAE): DIFFERENTIATION AND QUANTIFICATION OF OVERWINTERING AND SPRING MIGRATORY POPULATIONS IN NORTHERN MISSISSIPPI

By

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Helicoverpa zea, commonly referred to as corn earworm, has been a major pest of corn and cotton, along with many major crops grown in United States. Migration of this pest plays a major role in its distribution and successful survival. Part of the current resistance management strategy for transgenic crops, using non-Bt crops as refuges, is totally based on the movement of the adult populations between the Bt and non-Bt fields, and successful exchange of genetic material between the rare resistant and susceptible populations. To study the movement and migration patterns, and their implications in resistance management, suitable molecular genetic markers were comparatively selected, followed by a study of temporal variations in populations from north Mississippi. The dominant marker system Inter Simple Sequence Repeats (ISSR) was selected for the study based on higher polymorphism (5.0) and PIC (0.34) values compared to Simple Sequence Repeats (SSR) and Sequence Related Amplification Polymorphism (SRAP) marker systems. 53 ISSR loci were used in understanding the temporal variations in *H.*

zea. Percent polymorphism and heterozygosity levels showed variation among the twelve collections tested. Early spring putative migrants were distinct from the rest of the generations. Population differentiation was higher in the beginning of the season and then declined by end of the season (pairwise F_{ST} = 0.341, early in March and 0.025, late in August). Average heterozygosity levels recorded were 0.11, coinciding with 1993 and 2002 data.

The result of this study supports the existence of spring migration of adults and their contribution to the local gene pool. The extent of genetic exchange between the putative migrants and the local populations varied among three years. There is variation in population densities from migrants and local emergence in one of the three years. The results of this study indicate a need for continuous monitoring for genetic changes and their possible implications for resistance management in transgenic crops in Mississippi.

DEDICATION

I sincerely surrender all my efforts and hard work at the Lotus feet of The Mother and Sri Aurobindo.

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CHAPTER I

INTRODUCTION

Helicoverpa zea is a major pest of many agricultural crops such as corn, cotton, soybean and sorghum. Widespread in the United States, this pest is commonly referred to as corn earworm (CEW), "tomato fruitworm", "sorghum headworm", and also "cotton bollworm". Its counterpart, *Helicoverpa armigera*, is a major pest in Europe, Asia, Australia and Middle East, affecting a wide variety of crops. *H. zea* is active throughout the year in both tropical and subtropical climates. With a short life cycle and high dispersive ability, this pest spreads from the southern part to the northern parts of United States each summer. CEW is a generalized feeder both on crops and weeds with varying population dynamics (Hayes 1988). Survival and spread of the pest varies in different locations based on local weather patterns. The number of generations, based on the climatic conditions, greatly varies from one in Canada to seven in southern Florida and southern Texas. Together with *Heliothis virescens*, tobacco budworm, CEW constitutes the heliothine complex that can cause major damage and is responsible for significant economic losses. This complex is also responsible for development of some of the innovative strategies in the field of pest management. For many years, heliothines were considered major pests throughout the US cotton belt (Williams 2002).

A sixteen-year (1986-2001) survey of U.S. cotton yield losses due to insects averaged 8% yield loss, of which the bollworm and budworm complex accounted for 26% of the damage (Williams 2002). Among the many crops grown in the United States, cotton, soybean and corn are the main economic crops known for their productivity. The United States harvested 300 million tons of corn and 23 million bales of cotton in the year 2004 (NASS, 2005). Among the states, Mississippi is one of the major agricultural states in the southern United States with most of its agricultural production concentrated in cotton, corn, and soybean. 2003 statistics depict Mississippi as the 19th largest corn producer with 71 million bushels. The production fluctuates due to the weather, grower's decisions, and pest problems over the years. Cotton is another major crop grown in Mississippi, ranking 2nd in US with 2.1 million bales in the year 2003 followed by 2.3 million bales in the year 2004. As mentioned earlier, in addition to climatic and soil problems, these crops are frequently subject to heavy pest infestations. Crop production is always under intense pressure from pests damage in the form of quantity and quality losses. Quality loss can lead to rejection of loads of product, especially in the processing industries (green beans and sweet corn).

Over the decades, human endeavors to control insect pests have included many practices such as cultural, biological, mechanical and chemical control. However, use of insecticides has always been a preferred method due to its rapid action. Frequent applications, coupled with residual and other chemical properties of the insecticides, have resulted in many pests evolving resistance to many of the insecticide classes. One of the insecticide classes to which insects evolved resistance are synthetic pyrethroids, which were used on a large scale starting in the late 1970s (Stadelbacher et al. 1990, Kanga et

al. 1996, Brown et al. 1997, Hardee et al. 2001). Many new strategies were developed to overcome the problem of resistance in pests. Some of them include early planting, rotation of various classes of insecticidal mixtures, and limiting the use of pyrethroids to the early season (Luttrell et al. 1987, Graves et al. 1989). Even today, insecticides remain the most widely used practice against insect pests in agriculture, horticulture and medical fields. In agricultural production all over the world, bollworm is responsible for many insecticide related costs. In the United States alone, especially in the mid-south of the country, heliothines accounted for more chemical control than any other pest complex (Luttrell 1994). There has been progress in pest management over the decades, but insect pests have often survived by developing resistance to the chemicals used against them.

A new technology referred to as transgenic organisms (genetically engineered or genetically modified organisms / GMO's), with a foreign gene inserted into a species genome, was developed in the early 1990's that has been widely adopted in many parts of the world. Crops of economic importance are modified at genetic levels to enhance resistance against weeds, chemicals, insects and environmental stresses such as drought. These crops are widely accepted in many countries including North America due to the economic benefits to the farming community. Tomatoes that contained reduced levels of the cell wall softening enzyme polygalacturonase were the first transgenic plants to be commercially released, marketed as 'Flavr Savr' (Dunwell 2000). Most of the varieties grown to date have their genes modified for tolerance to herbicide or resistance to insects. Soybeans, corn and cotton are the major transgenic crops with tailored genes aiding in their survival against herbicides (Roundup Ready® against glyphosate; LibertyLink® cotton against glufosinate) and lepidopteran insects (Yieldgard® and Bollgard®).

Adoption rates were unprecedented, being highest for any new technology by the agricultural industry standards, reflecting the satisfaction and benefits from higher productivity and flexibility in crop management (James 2003).

Genetically modified cotton and corn, referred to as Bt corn and Bt cotton, have insertions of genes from the bacterium *Bacillus thuringiensis var kurstaki* Berliner that produce toxins, protecting them from lepidopteran pests (Perlak et al. 1990). The survival mechanism involves mortality of the immature stages, where delta-endotoxins act on the gut epithelium of the feeding larvae (antibiosis) (MacIntosh et al. 1990, Luttrell et al. 1999). These toxins are biodegradable and highly specific to their target insects. After rigorous experiments and scientific considerations, these transgenic Bt crops were first commercialized in the United States in 1996. Bt cotton and Bt corn are now distributed world wide except in some European nations and most of Africa, due to genetic concerns in the public and some non-governmental organizations (NGO's). The adoption of Bt crops has increased tremendously since commercialization in 1996. The overall acreage of Bt crops worldwide in 2001 was about 52.6 million hectares in 18 countries (James 2003). Transgenic Bt corn and cotton alone were grown on more than 162 million hectares worldwide as of 2006 (James 2006). New events of corn and cotton, recently released or in development that target *H. zea*, in addition to the previous released Bollgard I® and Yieldgard®, includes Bollgard II® (cry1Ac, cry2Ab), VipCot® (vip3a, Vegetative Insecticidal Protein & Cry1Ac), Yieldgard® plus, Yieldgard® VT corn, Widestrike®, Herculex®, Twinlink® and SmartStax® corn.

Evolution of insect resistance threatens the successful production of transgenic crops that produce the Bt toxins (Tabashnik et al. 2008). Simulations and laboratory studies showed that the insects could evolve resistance to transgenic cotton (Caprio 1994, Gould 1994, Roush 1994). The U.S. Environmental Protection Agency (EPA) mandated a high dose-refuge strategy in order to maintain susceptible pest populations and keep the frequencies of resistant alleles under check. The logic behind this strategy is that the rare adults (homozygous resistant) emerging from the Bt crops mix with numerous individuals (mostly homozygous susceptible) emerging from refuge, diluting (making heterozygous) the resistance alleles. Since Bt crops kill heterozygotes, an increase in resistance allele frequency is delayed (Gould and Tabashnik 1998, Gould 1998).

Some published work showed that the high dose strategy combined with refuges designed to control *Heliothis virescens* may promote resistance in *Helicoverpa zea*. It was also shown that *H. zea* couldn't be controlled to the desired levels with 1st generation transgenic cotton technology (Becheler and Mott 1997; Smith 1998). Though this technology was very useful against tobacco budworm (Williams 2000), CEW needed additional insecticide applications (Jenkins et al. 1992; Burd et al. 1999) due to the higher levels of tolerance to the Bt toxins (Stone and Sims 1993). The reasons for the differences in the effectiveness of the toxin are to some extent due to the temporal and spatial variation in the expression of the toxin in the plant as well the innate survival abilities of CEW (Gore et al. 2001, 2002; Adamczyk et al. 2001; Adamczyk and Meredith 2004). Also, the survival of the bollworm is greater on the transgenic varieties expressing lower levels of CryIAc compared to the varieties with higher expression (Adamczyk et al. 2001). This differential expression of the toxin in different varieties

might complicate current resistance management strategies. Recent commercialization of Bollgard II might be helpful in suppressing the populations of bollworm, with two genes, Cry 2Ab and Cry1 Ac, stacked into one variety. It was shown by Stewart et al. (2001) that the leaf tissues from Bollgard II® were more toxic than Bollgard® to second instar bollworm larvae. Pyramiding of two toxins in one system might be very helpful in suppressing the populations due to an increase in the strength of the toxin system (Caprio 1998), but it was also suggested (Gould 1986) that the pyramiding might not be immune to the development of resistance. Studies have shown that bollworms collected on Bt-cotton had higher LC_{50} values and are more variable compared to bollworms collected on non-Bt hosts (Luttrell et al. 2004). Also, the cultivars with higher doses might lead to more severe selection pressure (Gould and Tabashnik 1998).

Insect movement, which influences population mixing, causes a great variation in the survival abilities against conventional as well as transgenic insecticides. Dose mortality studies conducted on different individuals collected from different geographical locations showed a variation in sensitivity to CryIAc (Luttrell et al. 1999). Movement and feeding of CEW among multiple crops complicates the resistance management strategies. Simulations in the case of leafroller feeding on both apple and clover showed that even in the absence of complete cross-resistance, utilization of Bt genes in one crop could have a significant impact on the evolution of resistance to different Cry genes in other crops (Caprio and Suckling 2001). One of the features of *H. zea* survival, as mentioned earlier, is its wide host range (polyphagy) and its ability to move among different hosts and locations (Fitt 1989, Wolf et al. 1990). *H. zea* exploits multiple hosts concurrently or in succession. Larvae have been reported on more than 100 wild and

cultivated plant hosts (King and Coleman 1989). In addition to cotton, many agronomic crops provide a source of *H. zea* during much of the season in southeastern and mid-southern United States. It was shown that field corn is the most suitable host plant for *H. zea* (Gore et al. 2003). It is the most attractive host for oviposition during R1 and R2 (silking) growth stages (Johnson et al. 1975), which is typically in July (Bradley 1993) in the southern United States. During mid-to late summer (after silking, >R2), cotton turns out to be the most attractive host (Stadelbacher et al. 1986). When corn is most susceptible to CEW feeding, very few larvae will be present on cotton (Anonymous 1967).

Later in the year when a suitable host is not available, the populations survive on wild weed hosts. Craig (1998) determined that velvetleaf, *Abuliton theophrati* (L.), could support sufficient populations of bollworm and tobacco budworm and can be considered as a refuge for Bt cotton. Other non-cultivated hosts, including *Trifolium* spp, *Geranium* spp., *Vicia* spp., and *Lupinus* spp., were also attacked by CEW (Stadelbacher et al. 1986). In Mississippi, oviposition of CEW on spring hosts follows the flowering pattern (late March-early April, *Trifolium incarnatum*; mid-April to mid-May, *Geranium dissectum*; early June, *Medicago lupulino*; mid-June to early July, tasseling field corn, *Zea mays* (Stadelbacher and Martin 1980). Henry and Adkisson (1965) measured the movement of *H. zea* from alfalfa in the spring to corn in early summer, cotton in late summer as corn matures, and finally back onto alfalfa in fall, followed by diapause in August-October. Stadelbacher et al. (1986) listed 8 species as wild hosts for CEW in Mississippi, of which *Trifolium incarnatum* L. and *Geranium dissectum* L. were the major early-season hosts. After the first generation on wild hosts, adult moths move into corn at the time of silking

(highly attractive stage), with a few moving during whorl stage (Johnson et al. 1975, Lopez et al. 1978). Therefore, understanding the relationship of this pest with many hosts including the wild ones will help in achieving a better knowledge of population dynamics which in turn is vital in achieving successful control (Fitt 1989, Dent 1991).

Understanding the landscape ecology of CEW has become important in recent years due to the pest's ability to survive on a wide range of hosts. Adults use the landscape from wild to cultivated hosts as the season advances and then move back to wild hosts at the end of the season. Migration, movement of the individual from one place to another in search of a suitable host and habitats, has always played a key role in the insect's survival and pest status. According to Fitt (1989), the pest status of heliothines is attributed to their physiological, behavioral and ecological characteristics. The pest keeps moving between the fields during the season complicating management strategies. Studies have shown that inter-field movement can either spread resistance rapidly among fields or retard resistance development at the global level (Comins 1977, Caprio and Tabashnik 1992, Peck et al. 1999). Hardwick (1965) reported that CEW distribution included North America, South America, and the Caribbean islands. Migration studies have shown the ability of the pest to move every year from Mexico to Canada in search of suitable hosts and favorable climatic conditions. Some suggest that bollworm is a facultative migratory insect because of its ability to fly over long distances (Wu and Guo 1995, Guo 1997). Studies related to southward migration were also conducted, but have been unable to document this migration. One of the unique features of this pest that help in its survival as well achieving its pest status is its ability to overwinter in bad weather. CEW usually overwinters as a pupa in the fields where larva

developed and starts emerging with the arrival of suitable conditions early in the year. Snow and Copeland (1971) indicated that the diapause mechanism allows the insect to overwinter as pupae in soil as far as 45N latitude. Slosser et al. (1975) and Stadelbacher and Martin (1980) however, showed that the actual survival rate is less than 5% despite the survival up to 45N latitude. Some individuals, however, survive on wild hosts in the south due to continuous warm temperatures and hence have continuous generations in tropical and subtropical parts of the world.

Adult moths of *H. zea* are capable of flying long distances. Data indicate that flights of corn earworm moths commonly occur at altitudes above 1000 ft and may indicate mass migration (Callahan et al. 1972; Westbrook et al. 1998). Click (1965), in his review of collections of insects made with airplanes from 1926-57, concluded that the potential for migration was very good. He collected 35,826 insects during his flight from 6 – 4877 m altitudes and ca. 1% of them were lepidopterans. Spark et al. (1986) reported that the light traps placed on unmanned oil platforms located 32, 74, 106, and 160 km south of Louisiana shoreline in Gulf of Mexico showed evidence of long distance migration of *H. zea*.

Based on evidence that local emergence of individual adults occurs during a specific time period, researchers have suggested substantial early season migration of adult moths. Stadelbacher and Pfrimmer (1972) reported that moths were collected in light traps at least 30, 57, and 84 days before local emergence in 1967, 1968, and 1970, respectively. They concluded that the moths must have migrated into the Mississippi valley in late March and early April. Evidence shows that there was a major migration in 1981 of adult moths into areas around College Station 19 days prior to peak emergence

of the locally overwintered adults, and also 33 days ahead of the scheduled time, into Portland, AR during late March and April from Mexico (Hartstack et al. 1982). Hendrix et al. (1987) identified false mesquite, calliandra, and ape's earring, *Pithecellobium* spp., pollen on bollworm moths captured in Arkansas, although the nearest distribution of these plants is in south-central Texas. Lingren et al. (1993, 1994) found citrus and other pollen on bollworm captured in traps in Oklahoma and with the use of the estimated insect flight trajectories, estimated that the moths had migrated at least 700 km from southern Texas, Mexico, or the Caribbean region. Moths contaminated with citrus pollen were collected 661 km from areas of commercial citrus production in the Lower Rio Grande Valley, Texas in 1994 and 1996 (Westbrook et al. 1998). Studies from the Texas High Plains showed no significant relationship between moth catches in the fall to that of moths in the early season of the next year. This increase in trap collection densities might have been caused by the immigration of the moths (Parajulee et al. 2004). There is strong evidence regarding the northward migration of the adult moths. The adult moths that emerge early in the season in the south move north during the season and reach northern states by the end of August.

In the mid-south region of the U.S., the initial generation emerges from overwintering pupae in April and May (Anonymous 1967). Rummel et al. (1986) showed empirically that the onset of emergence of the overwintering generation varied from mid-April to mid-May in Texas Southern High Plains, and about 90% of overwintering moth emergence was reached by or shortly before mid-June. In Mississippi, local emergence may start around the mid-May and mid-April and the moths collected earlier in the season might be regarded as putative migrants (Schneider 2003).

Knowledge of insect population dispersal and migration helps in understanding the patterns of dispersal of pests and also in forecasting pest outbreaks (Loxdale et al. 1993). Knowledge of migration also aids in the development of simulation models that help in resistance management decisions. In insect models used to study the evolution of resistance and evaluate management strategies, movement of the pest is a key parameter (Caprio and Tabashnik 1992, Caprio 1998). Population genetic studies show that local moths differ from immigrants if there is little movement between the areas. CEW adults were reported to be homogeneous (Han and Caprio 2002; Hendrix et al. 1987) in terms of genetic structure, indicating high ratios of movement, hence a highly mobile nature. Migration studies, started in the early 70's, provided us with much needed information in understanding both inter-field as well as the intra-continental movement of moths throughout the year. This information augments pest management strategies in terms of changing the timing of insecticidal sprays, setting planting dates, and rotating crops. All the studies have provided information about the movement of the moths, but don't provide any information on the quantity of migration. Understanding migration patterns and the extent of movement helps in understanding population dynamics and in developing management strategies. Estimates of population genetics of the adult moths will help in estimating levels of gene flow and gene frequencies of both local and immigrant moths, which in turn will allow us to estimate the degree of mixing between these two populations.

The primary source of adult moths in the spring and early cropping season in the Southern United States is still controversial. Questions such as whether the moths are locally recruited or immigrants; are there any alternate hosts that are supporting the local

population during early season (Mueller and Phillips 1983) continue to vex entomologists. Based on recent studies conducted by Schneider (2003), it was shown that the two early-season peaks of adult moths, collected in pheromone traps represent two different populations. The first adult moths caught in traps were immigrants from phenologically advanced populations from lower latitudes. The second peak include locally produced individuals followed by a mixture of both populations in later catches. However, the relative magnitude of these two peaks is uncertain. There is no data to show the extent of overwintering in the state of Mississippi. Studies providing information about the levels of migration and the percent contribution of overwintering populations to the in-season moths would be a great asset in developing resistance management programs.

Recent additions to the knowledge of migration of this pest have provided much insight into the population dynamics, genetic structure and potential management strategies. Gould et al. (2002) used stable carbon isotope analysis to understand plant host use by CEW during the season. The study was based on the C3/C4 ratio in adult wings, which depends upon the food they fed upon during their larval stages. Cotton (C3), soybeans (C3), corn (C4), and sorghum (C4) are major food sources for the CEW during its lifecycle. It was clearly shown that the moths emerging early in the season are using C4 plants as the primary host and then move onto cotton later in the season. Previously it was thought that the sources of food for late season moths were cotton and soybeans, but this study has suggested that many develop on corn in the north and move south, followed by overwintering at the end of the year. Some of the studies that showed evidence for possible southward migration later in the season include one done by Pair et

al. (1987) where it was shown that the spring populations developed on corn in New Mexico and the Lower Rio Grande valley of Texas migrated north during summer (a heavy infestation was observed in conjunction with radar data) and moved back in September (fall) to the south, indicating reverse migration during fall into South Texas and Mexico from North Texas. Another study in this line includes Lopez et al. (1995) where there were high catches of CEW in the spring followed by a decline in mid-August, followed by dramatic increases in late August and early September that persisted until Mid-October. A recent study has suggested similar movement patterns in this region (Rennie 2003). Though much discussed, there are no data supporting the mixing of adult populations and transmission of genetic contributions to the next year. Again, there is no evidence whether these moths move further down to overwinter or to have continuous generations. This southward migration has alarmed the scientific community about the management strategies due to the continual survival and building up of resistant alleles over the season and the next season due to the emergence from the previous season diapausing moths. However, EPA's scientific advisory panel (SAP 2004) provided evidence that the reverse migration of moths does not have any effect on adaptation to Bt crops even when the percentage of corn and cotton planted with Bt technology is increased to 90% and 95%, respectively. In addition, there are many other host refuges such as peanut and soybean (Jackson et al. 2003) that act as a natural refuge and help in keeping the resistance allele frequency low. However, data are more applicable to diverse cropping systems as found in the southeastern states, where the study was conducted, and might not be the case in areas such as the mid-southern United States.

Studies in understanding the levels of migration of moths during early and late seasons help in gaining knowledge of the contribution of local and immigrant populations to the in-season populations. Also, quantifying the levels of gene flow, gene frequencies, and population differentiation (genetic structure) provides a better picture of population mixing. Again, this has great implications in delaying or accelerating the adaptation of CEW to the Bt technology.

Recent addition to the tools used in population genetic studies includes molecular markers that look into population differences at DNA level. These molecular tools, also referred to as molecular markers (named after their location in DNA), range from complex allozymes to more complex, but very informational, micro arrays. With increasing additions to the list of the markers used every day, use of polymerase chain reaction (PCR) based markers has increased over the years. With the introduction of PCR, many new techniques surfaced as an alternative to hybridization methods such as RFLPs. We will look into more details regarding these markers in the second chapter.

Our research in Mississippi from 2005 till 2007 included adult moth in-season movement data and their utilization in estimating population genetic parameters. This helped us gain knowledge of the movement of moths into the region during the early and late season, providing us with information about the migration of adults. Our research has not looked into the sources of populations based on C3 and C4 ratios, but used different molecular markers to gain stronger evidence from DNA of individual adult moths.

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CHAPTER II

COMPARISON OF PCR BASED MOLECULAR GENETIC MARKER SYSTEMS FOR POPULATION GENETIC STUDIES OF *HELICOVERPA ZEA* (BODDIE) (LEPIDOPTERA: NOCTUIDAE)

Abstract

Helicoverpa zea (Boddie) moths, collected from Monroe Co. in North Mississippi and Pennsylvania in 2005 were tested with three molecular genetic marker systems to determine the best system for population genetic studies. Forty individual moths caught using pheromone traps were studied using seven markers from each marker system. All the markers (microsatellites or Simple Sequence Repeats (SSR), Inter Simple Sequence Repeats (ISSR), and Sequence Related Amplified Polymorphism (SRAP)), were tested for genetic diversity and the ability to differentiate field-collected populations. For all marker systems used, polymorphic information content (PIC) was estimated. Dominant markers were corrected for small sample size and dominance by using Lynch and Milligan's correction. All three markers were good in explaining the genetic variation among the population. Of the three systems tested, two were dominant in nature and were more polymorphic when compared to the co-dominant microsatellites. Dominant ISSRs and SRAPs yielded 34 and 54 polymorphic bands when compared to 10 in case of

SSRs. PIC values were high in both dominant markers. ISSRs and SRAPs had PIC values of 4.86 and 5.29, respectively. On the other hand, microsatellites showed less diversity with a PIC value of only 1.29. Both the populations showed similar results with dominant markers exhibiting high polymorphism, with more polymorphic products. Coupled with high multiplex ratios and simplicity in conducting the assays, dominant markers don't require prior information on individual insect DNA sequences for developing them. They are easy to handle and cheaper to analyze large sets of populations. Based on convenience, low cost of investment, and ease in analysis, we concluded that dominant markers are a good alternative to co-dominant markers in studying the population genetic structure of *H. zea*.

Introduction

In the present world of advanced bioinformatics and data mining, there are many molecular markers used in different organisms to obtain information related to diversity. The targets of these markers differ from each other based on the goal of the study undertaken. Common types of markers used in these studies target mitochondrial DNA, ribosomal DNA, or nuclear DNA. Of these markers, nuclear DNA targeted markers are the most commonly used. Breakthroughs in technology have helped by increasing the array of DNA polymorphism assays for gene mapping and genome finger printing. Some of the commonly used markers include Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), and microsatellites (Lee 1995, Rafalski et al. 1996). Some of these are very laborious, inaccurate or have low consistency and poor multiplexing

output (simultaneous amplification of multiple targets of interest in one reaction by using more than one pair of primers). Due to the high multiplexing ratios (Vos et al. 1995), AFLPs are now widely used for various applications. However, the complexity involved in the procedure, including multiple steps and uneven marker distribution in the genome due to the use of restriction enzyme *Mse*I (Haanstra et al. 1999), is one of the main constraints to use of this method.

As mentioned earlier, markers used in today's research are much the same in plants as in insects, with some more or less applicable. Entomological research in the recent past utilized markers ranging from complex allozymes to more complex, but very informational, micro arrays. With increasing additions to the list of the markers used every day, use of polymerase chain reaction (PCR) based markers has increased over the years (Kumar et al. 2001a). With the introduction of PCR, many new techniques surfaced as an alternative to hybridization methods such as RFLPs. Advantages of PCR based methods include requirement of small amounts of template DNA, no need for radioactivity, and easy automation.

For this study with cotton bollworm, three PCR- based marker systems, SSRs, ISSRs, and SRAPs were included. SSRs uses nuclear genome with widely distributed simple sequence repeats (Hamada et al. 1982; Dietrich et al. 1992), and the variation can be easily visualized based on the polymorphism in the lengths of the amplified products (Tautz et al. 1986; Tautz and Renz, 1984). SRAPs involve using different primer combinations to amplify the DNA randomly. It involves two random primers, forward (17 bases) and reverse (18 bases), where the forward primer will have the first 10 bases as a filler sequence from the 5' end, followed by CCGG and then by three specific

nucleotides at the 3' end. In case of reverse primers, CCGG are replaced by AATT. The filler sequence, non-specific, will be different in both the primers. The rationale is that the exon's are rich in CCGG while non-coding intron's are rich in AATT, and therefore SRAP's amplify the sites within coding sequences rather than inter-genic regions. Seven primer combinations were used in this study (Li and Quiros 2001).

One of the drawbacks in using SSRs is the requirement of prior characterization of the sequences adjoining the repeats for designing the primers. However, it is a co-dominant marker and has an advantage of identifying multiple alleles and heterozygotes at a given locus. ISSR's were developed as an alternative to SSR to save time and expense involved in development and analysis. ISSR's generate many markers by targeting SSR loci, resulting in complex profile and generating additional polymorphisms, thereby providing an opportunity to screen many samples in a single gel, which can be either agarose or poly acrylamide based PAGE (Cekic et al. 2001).

These genetic markers are considered effective tools in making inferences about migration or movement studies as they represent selectively neutral characters (Black et al. 2001). Information derived from molecular markers in terms of gene flow estimates provide us with an estimate of dispersal rates (Krafsur et al. 2001). Genetic variation studies also help in providing an insight into the origins of individual populations that would be of a great value in identifying the population sources. Based on the fact that different markers reveal different levels of genetic variation, we tested all three markers with one bollworm population collected during the summer of 2005.

Dominant markers help in analyzing more than one loci at a given time and the primer utilized in amplifying a dominant marker amplifies many loci in one sample of

DNA. Co-dominant markers help in analyzing only one locus at a given time and hence the yield from a given primer includes one targeted product. Co-dominance means that both the alleles present at a locus are scorable, but in the case of dominance, only one allele can be seen as it masks the recessive allele. The results can be visually seen even before analyzing in complex computer based programs (microsatellites and AFLPs) as heterozygous alleles can be seen as two different bands in gel electrophoresis. Two markers selected for the study, ISSR and SRAP, were of dominant type. Unless stringent control measures are enforced, the markers loose their applicability. These dominant markers, when analyzed properly, are efficient tools in understanding the ecological and genetic structuring of populations (Black 1993, Lynch and Milligan 1994, Loxdale et al. 1996, Armstrong and Wratten, 1996, Brown et al. 1997, Vaughn and Antolin 1998, Pearson et al. 2002).

Among the selected markers, SRAP's (Li and Quiros 2001) are fairly new and previously have not been used on arthropods. These markers are reliable and easy to handle just as ISSR. Many studies were done on plant genetic diversities using SRAP's (Budak et al. 2004). On the other hand, SSR's (Perera et al. 2007) and ISSR's have been widely used in insect studies (Kumar et al. 2001b; Nagaraju et al. 2001).

Field collection of samples

Adult moths were collected every week from nine Hartstack pheromone traps placed in Monroe Co. in northern Mississippi from March to October of 2005. The moths collected from different traps were pooled by date into four different populations for analysis. Moths (40 adults) from July 2005 were used for this study (Fig. 2.1 & 2.2). Seven primer combinations from each marker system were used to test the moths for genetic differences.

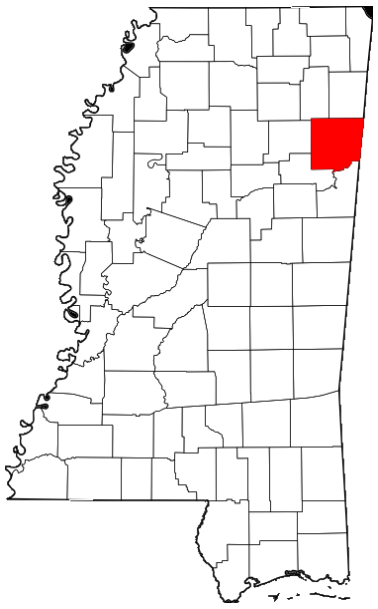

Figure 2.1 Study site where all the *H. zea* moths were collected

Figure 2.2 Location of Hartstack traps in Monroe Co. of North Mississippi.

DNA extraction and amplification

Adults were selected randomly from the pool of well-preserved moths (–80°C), collected during summer season, for extracting the genomic DNA. Only the thoracic segment of the moth was used in extraction to avoid unwanted debris from the stomach and head that might interfere with the amplification process. Similarly, care was taken to exclude scales as they interfere with the extraction process (personal communication from Juliet Tang, Life Sciences & Biotechnology Institute (LSBI), Mississippi State University (MSU). The MasterPure ™ reagent system (Epicentre Technologies, Madison, WI) was used for all extractions. The DNA concentration in the extracted samples was checked using a NanoDrop® ND-1000 spectrophotometer, located at LSBI, MSU. All extracted

samples were stored at –80°C until further analysis. The extraction methodology was common for all marker systems used in the study.

Table 2.1 Primer sequences of the Inter simple sequence repeats (ISSR) used in study

UBC Primer #	Primer Sequence
816	CAC ACA CAC ACA CAC AT
818	CAC ACA CAC ACA CAC AG
825	ACA CAC ACA CAC ACA CT
826	ACA CAC ACA CAC ACA CC
827	ACA CAC ACA CAC ACA CG
828	TGT GTG TGT GTG TGT GA
861	ACC ACC ACC ACC ACC ACC

Table 2.2 Sequence related amplification polymorphism (SRAP) primers used in the study

Forward Primer	Reverse Primer
TGAGTCCAAACCGGATA	GACTGCGTACGAATTTGA
TGAGTCCAAACCGGATA	GACTGCGTACGAATTAAC
TGAGTCCAAACCGGATA	GACTGCGTACGAATTGCA
TGAGTCCAAACCGGAAT	GACTGCGTACGAATTGCA
TGAGTCCAAACCGGACC	GACTGCGTACGAATTTGC
TGAGTCCAAACCGGACC	GACTGCGTACGAATTGAC
TGAGTCCAAACCGGACC	GACTGCGTACGAATTAAC

Protocols used in amplification differed slightly for each marker system. The concentration of the chemical reagents used as well as the conditions for PCR reactions differed for each system. The primers used in ISSR, SRAP, and SSR protocols are shown in Tables 2.1, 2.2 & 2.3, respectively. ISSR primers used in this study were synthesized by University of British Columbia Biotechnology Laboratory (Vancouver, Canada) (primer kit no. 9), and used previously in a similar study (Looft et al. 2007). Perera et al. (2007) developed SSRs for *H. zea*, where as SRAPs were developed by Li and Quiros (2001) for their use in plants. In all cases, DNA concentrations were measured to make sure that there was enough DNA template in each sample needed for the amplifications.

Polymerase chain reaction mixtures were adjusted such that at least 30-50ng of DNA was present in the final reaction. For SSRs and ISSRs, the reaction volume of 25

µl included 1x taq polymerase PCR buffer, 0.4mM dNTPs, 2.0mM magnesium chloride, 0.6 µM of primer, 1.0 U of platinum *Taq* DNA polymerase, and 30-50 ng of total DNA (Looft et al. 2007). The reaction steps for ISSRs include initial denaturation for 4 min at 95°C, followed by 35 cycles of 90s at 95°C, 30s at 50°C, 120s at 72°C, and a final extension of 4 min at 72°C.

The SSR reaction included initial denaturation for 4 min at 95°C, annealing at 60°C for 1 min, and followed by 26 cycles of 95°C, 60°C, and 68°C for 30s each. SRAP's reaction mixture of 20 µl included 1.5 µl of 10x PCR buffer, 1.0 µL of 25mM $MgCl_2$, 1.0 µL each of 10 µM fixed and arbitrary primers, 1.0 µL of 10 µM dNTPs, 0.35 µL of 5U *Taq* polymerase and 1.0 µL of 50 to 80ng genomic DNA. The PCR conditions specific to this marker are as follows: initial denaturing at 95°C for 4 min followed by 35 cycles at 94°C for 45s, 35°C for 45s and 72°C for 1 min with a final extension step at 72°C for 7 min (Li and Quiros 2001).

All amplification reactions were performed in a Mycycler® thermal cycler (Biorad Labs, Hercules, CA). Repeating the amplification reactions always tested reproducibility of the markers. Also, a negative control without DNA was included in all runs to ensure purity. All amplified products from the three markers were analyzed on 2% agarose gels. A 10K bp ladder (Hyperladder I, Bioline Cat. BIO 33025) was used as a size standard to measure the size of the fragments. The size of amplified products ranged from 200-3600 bp. Gels were visualized by means of ethidium bromide staining of DNA. The gels were digitally scanned as TIFF (tagged image file format) files by gel documentation system (GDS7500, Kodak).

Table 2.3 Microsatellite (SSR) primer sequences used in the study

Locus #	Forward Primer	Reverse Primer
HZMS 1-4	CAAGTGATAAAAGACGCCGAAGAT	GTTGATCGTCAAGGAAGTGGCTAT
HZMS 3-1	CAGTAGTTCCTGAGATTAGCGCGT	ATCACGTTCTCGAAAAACATTGCT
HZMS 3-4	GGTCAAGATTCGTGCCGATAACTA	TTTTCGGTTCAGTGGCTTGTAGTAG
HZMS 4-3	ACTTTCCGCATCCGATTAAAATGT	CAAATCGGACCAGTAGTTCCTGAG
HZMS 3-11	ACTTCAAAGTTCGATTCTTGGGAT	GCTCAAAGAGGACTACGTAGCTGA
HZMS 3-41	AAATTTCAACCAAATCGGTCTAGC	TGGCCGAACTATAATATCTTACTTCCTA
HZMS 3-48	GGTGAAATGGAAATTGTTATCTATCCC	TCAGTCCAGTGGTTTAGACGTGAA

Data analysis

The gels were scored for the presence (1) or absence (0) of bands or loci. Care was taken to score only those bands that were readable. All ambiguous bands were ignored and excluded from our analysis. In case of ISSR's and SRAP's, reproducibility of the bands were checked by repeating the protocol twice with the same samples, and only those bands that were reproducible were included in our final evaluation. In case of SSR's, the amplified products were further subjected to fragment analysis by means of Beckman Coulter's CEQ 8000 Genetic Analysis System, located at LSBI, Mississippi State University. The fragments were then scored as homozygous or heterozygous, based on presence of two bands or one band, for a given locus in all individual samples.

In judging the performance and the utility of the marker system in determining the polymorphisms and genetic diversity among the *Helicoverpa zea* populations, we utilized the parameter referred to as Polymorphic Information Content (PIC) (Equation 2.1).

The respective indexes are measured as follows.

$$PIC = 1 - \Sigma f_i^2 \qquad (2.1)$$

where, f_i is the frequency of the *i*th allele (Weir 1990). Considering the number of alleles at a locus along with their relative frequencies in a given population, an estimate of the discriminatory power of a marker can be obtained (Vuylsteke et al., 2000).

A total of 40 individual moths were used in evaluating the effectiveness of these markers. Seven primer combinations in total were used from each marker system for final analysis. All the adult moths used in this study were earlier collected from pheromone traps during the 2005 crop season. These results were compared to another population of 40 individuals from the same year, sent by Dr. Shelby Fleischer from Pennsylvania, collected as a part of sweet corn monitoring program. The project information regarding monitoring can be viewed at http://www.pestwatch.psu.edu/sweetcorn/tool.html.

Results

The two adult populations studied by using three different marker systems gave similar results in terms of genetic diversity. Seven primer combinations from each marker system were tested for their applicability in population genetic studies of *Helicoverpa zea*. The number of bands obtained was very consistent with microsatellites and SRAPs, with more variation using ISSRs.

Microsatellites

Seven SSR primers produced 12 and 13 alleles in the populations from Mississippi (MS) and Pennsylvania (PA), respectively. The band sizes varied from 97 to 126 bp. Of the 40 MS adult moths tested, nine loci were polymorphic, exhibiting different levels of heterozygosity among the moths tested. In other words, of the 12 amplified products obtained, 9 were polymorphic with 1.4 average products per primer (Table 2.4). This was less than the other two markers tested. The other two markers tested showed many polymorphic bands (35 and 41 with ISSR and SRAP's, respectively). Similar results were obtained with individuals from Pennsylvania. Amplification revealed 10 polymorphic bands with 1.4 polymorphic products per primer tested. Polymorphic information content (PIC) values for SSRs were low compared to the other two marker systems. In case of SSRs, these values were 0.21 and 0.23 for moths from Mississippi and Pennsylvania, respectively (Table 2.6 & 2.7). PIC, which measures information content as a function of a marker system's ability to distinguish between genotypes varied among the combinations from 0.14 in HzMS1-4 to 0.49 in HzMS3-48, with an average of 0.21.

Inter simple sequence repeats

Individuals tested for polymorphism with ISSR's revealed 35 amplified products with 34 of them being polymorphic (Table 2.4 & 2.6). Seven primers tested against 40 individual moths were very effective in terms of differentiation and polymorphism. The percentage of polymorphism was close to 100. Five polymorphic bands were revealed per primer used in the study. Average heterozygosity for polymorphic loci was consistently high in populations from both Mississippi and Pennsylvania. The PIC values for UBC primers ranged from 0.28 for 827 to 0.42 for 850. The average PIC values were high in both locations, being 0.34 and 0.40 in MS and PA, respectively (Table 2.5 & 2.6).

Sequence related amplified polymorphism

SRAP's, a fairly recent development and not yet utilized in insect studies, were tested in our study to not only see if they are applicable, but also to see if they are better than the typical markers used in this type of study. Results obtained with these markers were comparable with ISSR's, both being dominant. The polymorphism was high and with greater differentiation than SSRs. Seven primer combinations utilized in this study yielded 41 (Table 2.4 & 2.5) and 55 amplification products from individuals sampled from MS and PA, respectively. The percent of polymorphic product per primer ranged from 5.29 to 7.71 with 37 and 54 polymorphic bands in MS and PA populations, respectively.

Table 2.4 Summary of assays of each marker system performed on 40 moths from Mississippi.

Marker system	Total no. of assays	Total no. of products	Percent polymorphism	Polymorphic information content
SSR	7 (primer pairs)	12	75	0.21
ISSR	7 (primers)	35	97	0.34
SRAP	7 (combinations)	41	90	0.30

Table 2.5 Summary of assays of each marker system performed on 40 moths from Pennsylvania.

Marker system	Total no. of assays	Total no. of products	Percent polymorphism	Polymorphic information content
SSR	7 (primer pairs)	13	77	0.23
ISSR	7 (primers)	35	97	0.40
SRAP	7 (combinations)	55	98	0.41

The PIC values ranged from 0.04 to 0.40 for primer combinations 42 and 16, respectively. The average PIC value was higher than SSRs at both MS and PA with values being 0.34 and 0.40, respectively (Table 2.4 & 2.5). Our results show that these markers can be used for this kind of diversity study.

Discussion

H. zea, commonly referred to as cotton bollworm or corn earworm (CEW), is a major pest affecting cotton, corn and some 30 other crops in the United States alone. Researchers ability in studying the population genetics of this particular pest is restricted or limited due to the non-availability of suitable genetic markers. Understanding the population genetics of *H. zea* would help us to improve and develop superior integrated pest management strategies in the future. Previous research indicate that the population genetics of CEW are difficult to study due to its wide geographic range, polyphagy, complex migratory behavior, multiple mating, diapause, and multivoltinism. Development or testing new genetic markers against CEW will help us in understanding critical population parameters such as genetic structure.

Previously, there were no microsatellites or SSR's specifically developed for CEW. SSR's developed for *Helicoverpa armigera* (Tan et al. 2001; Ji et al. 2003; Scott et al. 2004) were tested against CEW (Grasela and McIntosh 2005) with limited results, as the CEW were not from field populations. The SSR's developed by Perera et al. (2007) showed some promise in their application to population genetic studies and mapping. To include more genetic markers for this kind of research, we have tested three different markers for their variability in field populations to facilitate analysis of the genetic diversity among the CEW populations. Three markers used in this study included SSR's, ISSR's, and SRAP's. Results indicated that all three markers are a good fit for understanding the basic population diversity estimates. However, the use of SSRs is more complex and costly in time and money than ISSR and SRAP, and so the dominant marker systems are preferred for this type of study.

The dominant markers, ISSR and SRAP, are easy and cheaper in developing, as no prior knowledge of the DNA sequence is needed, and are not designed based on the sequence. Once developed, the primers can be used in amplification of the target DNA without problem and the amplified products can be easily viewed for further analysis on regular gels. One can use starch based agarose gels as well as polyacrylamide based gels in scoring the alleles.

In this study involving three markers and forty adult moths from Mississippi and Pennsylvania, we have shown dominant markers are a possible alternative for population studies of *H. zea*. Microsatellites for CEW were recently developed (Perera et al. 2007) for these kinds of studies and proved to be a good marker to study population diversity in this pest. In published studies on SSR and ISSR's, the markers were tested against populations of CEW collected from Mississippi, Texas, Alabama, and Arkansas, to see how they were related (Perera et al. 2007 and Looft et al. 2007). In our study, we focused on populations from two different states, namely Mississippi and Pennsylvania for within population comparisons only. All the primers used in this study except SSR's were developed for other organisms, but were tested against CEW for their applicability. ISSR's (100 primers) were tested previously (Looft et al. 2007) against some CEW from Texas to Pennsylvania for their ability in differentiating the populations. Eight primers were identified as the best ones in their applicability to CEW population genetic studies. We have used seven of these eight primers in our study of comparisons.

Our study indicates that, for both the populations tested, the PIC values for dominant markers were comparatively higher than for co-dominant markers. The mean number of polymorphic products obtained per primer was higher for ISSR's (4.86) and

SRAP's (5.29) than for SSR's (1.29). The PIC value of 0.21 in case of SSR's was low when compared to a PIC value of 0.34 for ISSR's. Similarly, the mean PIC value for the SRAP markers used in the study was 0.30. SSR's revealed a low overall percentage of polymorphism (Table 2.6 & 2.7). For the seven primers tested from each marker system, SSR's revealed only 10 polymorphic bands compared to 34 and 54 in case of ISSR's and SRAP's, respectively. Newly developed PCR-based marker systems are playing a significant role in analyzing the genetic diversity of large number of insect species (Kumar et al. 2001a). Higher levels of polymorphism and better multiplex ratios, in addition to the simplicity of PCR-based assays, make them an attractive alternative in obtaining intraspecific genetic variation, especially in species like *Helicoverpa* spp. where there is no prior sequence information. These marker systems detect polymorphism by assaying a subset of the total amount of the DNA sequence variation in the genome, except in case of SSR's. Inter Simple Sequence Repeat markers are in use in many organisms and are in demand, as they are known to be abundant, reproducible, polymorphic, and highly informative along with ease to use (Zietkiewicz et al. 1994, Bornet and Branchard 2001, Bornet et al. 2002). ISSR's uses Simple Sequence Repeats that are ubiquitous, abundant and highly polymorphic, with tandem repeat motifs composed of 1 to 7 nucleotides, and are present throughout the genome. It was shown here that SRAP's are equally effective to ISSR's.

Assessing genetic relationships among the populations can be affected by the number of markers used as well their distribution in the genome (Leberg, 1992; Nei, 1978). For precise estimates, markers that are randomly distributed and span the whole genome should be selected. This is usually true and possible when a molecular marker

linkage map is available. As this is not available for *H. zea*, the best alternative was to test different marker systems and estimate the efficacies of each marker system. Based on convenience, cost of investment, and ease of obtaining and analyzing, we conclude that dominant markers, namely ISSRs and SRAPs are good marking systems for population genetic studies of *H. zea*.

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CHAPTER III

TEMPORAL VARIATION IN GENETIC STRUCTURE OF CORN EARWORM, *HELICOVERPA ZEA* (BODDIE) (LEPIDOPTERA: NOCTUIDAE) IN NORTHERN MISSISSIPPI EVIDENCED FROM ISSR MARKERS

Abstract

Population genetic structure of *Helicoverpa zea* in North Mississippi was studied using newly developed molecular genetic markers. Temporal variation in adult moths from three consecutive years (2005-2007) was evaluated with ISSR markers. The main emphasis of the study was on variation in the early spring populations due to differentiation between putative migrants and locally emerged adult generations. Adult moths were separated into four groups based on collection timings in each year. The results were corrected for small sample size and the dominant nature of ISSR markers. Lynch and Milligan pairwise F_{ST} values were higher between the first collection and second collections, followed by a decrease between later collections in 2 of 3 years. This result is compatible with the hypothesis that early season moths are immigrants. Previous research suggests that early spring migration of adult moths is from more southern populations. Consistent with previous studies, heterozygosity levels are near 0.1.

Introduction

Commonly referred to as cotton bollworm or corn earworm (CEW), *H. zea* (Boddie) is one of the most economically important pests in the United States, affecting a wide range of agricultural hosts including cotton and corn. CEW, being highly adaptable to the weather conditions and polyphagous, affects many fields in the southern United States. Some of the characteristics responsible for its success as an exploiter of agronomic systems include polyphagy, multivoltinism, high mobility, high fecundity, and the ability to enter diapause (Fitt 1989). Together with tobacco budworm (*H. virescens)*, jointly referred to as heliothines, the larval stages of CEW cause considerable damage in United States. One report showed that the total management cost in 2001, including losses in yield, from the heliothine complex was about 224 million dollars (USDA 2002). As mentioned previously, immature stages feed on a variety of crops, with greater preference for corn in its silking stage (Isley 1935, Johnson et al. 1975) than other hosts. It's feeding on corn is more prevalent in North America than other regions (Hardwick 1965). It was also responsible for more applications of insecticides in cotton than any other insect pest (Luttrell 1994). Due to frequent selection during the mid-season from insecticides used in corn and cotton fields, data show that CEW has evolved resistance to many important insecticides (Pietrantonio et al. 2007).

Manipulation of the insect environment by using IPM practices helps minimize damage from *H. zea*. Combining cultural, mechanical, and chemical methods to control the population helps keep populations in check. These tactics include changing the timing of insecticide applications. Synthetic pyrethroid applications, to which CEW developed resistance, were limited to the early season in the southern United States to

manage resistance to this group of insecticides, by keeping the selection pressure and resistant allele frequencies at fairly low levels (Graves et al. 1989). With the onset of resistance in heliothines (primarily tobacco budworm) to several classes of insecticides including carbamates, chlorinated hydrocarbons, and organophosphates (Graves et al. 1963; Wolfenbarger and McGarr 1970; Harris 1972; Wolfenbarger et al. 1973; Plapp and Campanhola 1986; Luttrell et al. 1987), achieving acceptable control with conventional insecticides was difficult and expensive. Recently, genetically modified or transgenic crops, with a gene transferred from *Bacillus thuringiensis*, have performed better in controlling these insect pests.

Bt corn and cotton, expressing a gene from *Bacillus thuringiensis* that produces a protein with insecticidal activity, were commercially released in 1996 to control some of the major lepidopteran insect pests such as CEW, tobacco budworm, and European corn borer. The commercial release of Bollgard® varieties in 1996 provided cotton producers with an effective means to manage heliothines (Hardee et al. 2001). While Bollgard® cotton provided excellent control of TBW, in 2003, U.S. producers planted Bollgard® cotton on nearly 2.5 million hectares with foliar applications of insecticides targeting heliothines, mostly CEW, still applied on more than 1.2 million hectares of the Bollgard® cotton (Williams 2004). Heliothines were still the most damaging pests in cotton in 2003 with the complex infesting almost 2.1 million hectares, causing a 1.4% reduction in yield (Williams 2004). In the United States, genetically modified cotton (herbicide tolerance and/or insect tolerance) was planted on 6.8 million hectares in 2002, which was approximately 12% of the world's genetically modified crops. Bt cotton accounted for over 2.3 million hectares of this crop. Globally, the United States grows 57.7 million

hectares of the world's transgenic crops with herbicide tolerance and insect resistance traits (Lawrence 2008).

Transgenic technology came with the potential for rapid evolution of resistance in the targeted pests. Many laboratory studies were conducted to understand the mechanisms as well as speed with which the individuals might develop resistance to the Bt toxins. Lepidopteran pests such as diamondback moth, *Plutella xylostella* (L.); tobacco budworm; beet armyworm, *Spodoptera exigua* (Hübner); and the Indian meal moth, *Plodia interpunctella* (Hübner); (Gould et al. 1992, Tabashnik 1994) were artificially selected for *Bt* resistance. The first evidence of field-evolved resistance to foliar applications of Bt toxins came from Hawaii, in diamondback moth (Tabashnik 1994).

Furthermore, several studies show that transgenic crops are not without risk of the evolution of resistance (Caprio 1994, Gould 1994, Roush 1994). Convincing data from studies on the evolution of resistance to Bt crops helped the EPA (Environmental Protection Agency) in enforcing strong Insect Resistance Management (IRM) regulations such as the mandatory adoption of refuges, a non-Bt crop managed to produce susceptible moths. The theory behind the refuge strategy is that most of the rare homozygous resistant (RR) pests surviving on Bt crops will mate with abundantly available susceptible (SS) pests from refuges of host plants that lack Bt toxins. If inheritance of resistance is recessive, Bt crops will kill hybrid (RS) offspring resulting from mating. This whole process helps in slowing down the evolution of resistance (Gould 1998; U.S. EPA 1998; Tabashnik 1994; Tabashnik et al. 2003).

Studies on refuges indicate that they can significantly slow down the rate of resistance development if the refuge produces 500 or more susceptible insects for every resistant insect that develops on a transgenic plant (Gould 1998). A high-dose desired in the high-dose refuge strategy is the expression of at least 25 times the concentration of Bt toxin needed to kill 95% of susceptible insects (Tabashnik and Croft 1982, Gould 1998, SAP 1998). While currently deployed transgenic plants provide a high dose to TBW, they do not meet the high-dose requirement for CEW, as they do not kill more than 95% of the heterozygotes (EPA 2001). Gore et al. (2003a) looked at the effects of alternate hosts (field corn, grain sorghum, soybean, and non-Bollgard® cotton) on adult survival, larval development, and susceptibility of subsequent generations to Bollgard® cotton. Their study showed that the success of IRM strategies with refuges may be directly influenced by the availability of early season alternate hosts. Similarly, other studies show that resistance may evolve rapidly in areas with high Bollgard® cotton acreage and less refuge or non-Bollgard® crops (Hardee et al. 2001, Burd et al. 2000).

Migration is a key parameter in modeling the evolution of resistance (Caprio and Tabashnik 1992, Caprio 1998). Interfield movement can either spread resistance rapidly among fields or retard resistance development at a global level (Comins 1977, Caprio and Tabashnik 1992, Peck et al. 1999). Movement leads to a decrease in differentiation in the populations due to gene flow. Egg stages are more differentiated than the later mobile stages with gene flow (Han and Caprio 2002).

Previously, many research articles were published on migration of CEW, some with the help of markers such as citrus pollen, some based on radar studies, and some based on isotopes (Johnson et al. 1975; Westbrook et al. 1997; Rennie 2003). Studies

have strongly supported the conclusion that CEW moves north every year. One unique approach used in documenting northward movement was one based on the pollen that adhered readily to the insect's body parts. As an insect feeds on such plants, adults get marked naturally with the pollen (Kapp 1969). Knowledge about the distribution and timing of flowering of most plants helped in establishing the origin of the insects (Hendrix et al. 1987, Hendrix and Showers 1992, Lingren et al. 1994). Hendrix *et al.* (1987) identified pollen of two Mimosoideae legumes, *Pithecellobium* spp. (Ape's earring or Texas ebony) and *Calliandra* spp. (false mesquite), whose pollen were stuck to the eyes and proboscis of CEW that were captured during spring in Arkansas. These plant genera occur only in southern Texas, indicating that the adults moved at least 1000 km from their origin. Also, weather patterns and radar studies have helped in identifying the trajectories of several lepidopteran pests (Lingren et al. 1994, Westbrook et al. 1997, 1998). Wind trajectories were well correlated with the displacement pattern of CEW that were marked with *Citrus* pollen and *Lycopodium clavatum* spores, helping in understanding a flight range of at least 660 km in Texas (Westbrook et al. 1998). Another example in this area includes the study done on black cutworm by Showers and Whitford (1989). They showed strong evidence of migration by a mechanistic link between synoptic southerly winds and the recapture of marked black cutworm moths, *Agrotis ipsilon* (Noctuidae), far from their release site (≈1000 km). In a study by Pair et al. (1987), CEW migration was shown from Mexico and the Lower Rio Grande Valley into the Texas High Plains, in synchrony with local emergence and high moth trap catches.

There has been debate about southward migration of *H. zea* populations towards the end of the growing season. Radar profiles of airborne insects, in conjunction with higher catches in pheromone traps in Texas, showed the possibility of populations in the High Plains completing a reverse migration late in the growing season (Pair et al. 1987). With the commercialization of transgenic Bt cotton and corn in 1996, and with many others to come in the future, there has been an increased focus on the issue of migration, particularly southward as it could have significant impact on the rate of resistance development to the Bt toxin.

Southward migration from non-Bt corn helps in increasing the overall refuge levels for susceptible populations that would reduce the rates at which the resistance evolves. Rennie (2003) hypothesized that CEW moves north early in the summer and feeds on corn, followed by reverse movement to the south to feed on cotton or wild hosts. The concentration of the naturally occurring isotope, $_{13}C$, which gets integrated in wing cuticle of a moth during its development, acts as an indicator of the photosynthetic pathway (grass/corn=C_4, cotton/soybean=C_3) of the plant that it feeds on. Analysis of adult moths caught in pheromone traps (early fall of the year 2000) in Brazos River Valley, Texas, showed that individuals (40-100%) had developed on C_4 hosts rather than the C_3 cotton grown in that area. This study also showed differences in the wing lengths with longer wings in adults that fed on C_4 hosts as larvae than on C_3 hosts.

Despite a very low frequency of resistant genotypes in the field (Jackson et al. 2003), bollworm larvae have a 5-25% rate of survival on Bollgard plants (Gore et al. 2003b). In addition, previous studies have indicated that a bollworm strain with genetic tolerance to Bollgard (Cry1Ac) has a low level of tolerance to Bollgard II (Cry1Ac and

Cry2Ab) plants grown in the greenhouse (Jackson et al. 2000). Having higher initial tolerance in bollworm to *Bt* (Luttrell et al. 1999) than tobacco budworm and pink bollworm, there is concern among the scientific community regarding the evolution of resistance in this species.

In this current study, we examined *H. zea* populations, collected from Monroe Co. of North Mississippi, for temporal variations in genetic structure. The early spring populations, putative migrants from the south were compared to later generations in the same year for temporal variation and genetic structure. Putative migrant adults, collected early in the spring (March), and local populations over the season, were tested using dominant genetic markers. Efforts were made to understand the genetic diversity over a period of three years. Genetic variation between generations was examined to determine changes in genetic structure over time.

The goal of the study was to differentiate the putative migrants from the locally emerged overwintering adults and to determine the source of mid-summer populations. Genetic contribution of the early spring migrants to the local populations was studied over three years with Inter Simple Sequence Repeats. This gave us an idea of present diversity levels, population differentiation, adult movement and mixing in the area. The anticipated results will give us an idea of *H. zea* diversity and will help us design the pest management strategies as well as refuge recommendations.

Materials and Methods

The methodology followed for this study was similar to what was done for the earlier study, as explained in Chapter II.

Collection of in-season adult moths

Based on previous research regarding moth emergence and possible migration of adult moths from the south (Schneider 2003), wire Hartstack traps (Texas cone 50-25) (Hartstack et al. 1979) were setup in Monroe Co. in Mississippi. We have not collected any adult moths near the Mississippi State University campus or nearby research fields to reduce the possibility of capturing adults that were released in the past for other studies. Nine traps with CEW-specific pheromone lures were used to capture adult males. The pheromone lures used in each trap were replaced every two weeks. Traps were checked every week for fresh moths and all the collections were transported to the lab for storage. Only those adult moths that were well preserved, not damaged by biotic or abiotic factors, were used for our studies, as they would provide a consistent amount of non-degraded DNA. Some of the features we used in making the selection included intact scales, green eyes, and soft body. All the adult moths that met the mentioned criteria were saved at -80C to preserve the DNA until extractions were carried out at the end of summer. Adult moth collections were started from late March and continued until October for all three years. Care was taken to avoid mixing of moths from different time periods.

The early collections were assumed to be migrants from south, followed by later generations from both local and the offspring of both local and non-local moths. Moths

collected prior to mid-April were considered immigrants and then the collections made from May were considered a mixture of both (Schneider 2003). The adult moths from different time periods were saved separately. Efforts were also made to collect locally overwintered adult moths as they emerged by using eclosion traps. These traps were set up in the same county with some around the county to get as many moths as possible. These moths are considered the progeny from the previous year's populations that went into diapause. Usually these overwintering populations emerge the following year with the arrival of warmer temperatures, around mid-April to mid-May (Schneider 2003).

Collection of overwintering moths

In general, late season moths from August and early September undergo diapause as pupae during the cold winter temperatures and emerge the following year in late May (Stadelbacher and Pfrimmer 1972; Stadelbacher and Martin 1980). These late season adults survive on wild hosts in the absence of a cultivated crop (Stadelbacher et al. 1986). Efforts were made to capture adults from these alternate hosts. In the year 2005, we set up eclosion traps made-up of cloth. These traps were spread across Monroe Co. in places where there were many alternate hosts that might have supported their survival the previous year. The traps were checked every week until early June. In the fall of 2005, we planted chickpea plots to attract the adults such that some of them could diapause in the same location. These plots were located at the Black Belt Branch Experiment station, Brooksville, MS. This time, eclosion traps made out of metal wire that looked like cone traps, but placed on the ground instead of on a pole, were used.

The chickpea study had more than 20 traps, enough to cover the whole plot (11 1.0m center rows by 27 m long) of chickpeas, with plant population averaging 3.0 per row-meter. Our study was totally based on pheromone trap collections from four time periods in a year.

Extraction and amplification of DNA

The procedures used in extracting and amplifying DNA, using specific PCR conditions, were similar to the methods in Chapter II. Instead of using all three types of markers as in the previous study, we used ISSRs for this study to analyze all the adult moths from four time periods and three years, 2005-2007. Forty adults and eight primers were used for each time period. All the amplified products were then subjected to electrophoresis on 2% agarose gels. The gels were then stained in ethidium bromide and the gel pictures were saved for scoring followed by data analysis.

Data analysis

Each individual was scored at each locus as present (1) or absent (0). This scoring was done across all polymorphic loci to create a binary matrix. Only bands that were clear and unambiguous were selected for scoring. A locus was considered polymorphic if the frequency of the most common allele did not exceed 0.95. All frequencies were estimated by using the program RAPDBIOS 2.0 (Black 1997). This program automatically employs Lynch and Milligan (1994) correction needed for estimating allele frequencies from dominant markers such as ISSRs. Genetic diversity was calculated using BIOSYS-2 (Swofford and Selander 1981). The genetic diversity within the populations was quantified as the percentage of polymorphic loci estimated from allele

frequencies and Nei's (1978) unbiased heterozygosity (H_E). All the calculations were done under the assumption of Hardy-Weinberg equilibrium.

Program RAPDDIST 1.0 (Black 1997) was used in calculating genetic distances among populations based on Nei's (1978) unbiased genetic distance. Population differentiation and gene flow estimates among populations were computed by using the RAPDFST program 4.0.1 (Black 1997). This program gives Weir and Cockerham's (1984) F_{ST}, corrected for small and unequal sample sizes. Three different types of F_{ST} are calculated from this data, Fisher's F_{ST}, Weir and Cockerham's theta (θ) and Lynch and Milligan (LM) F_{ST}. In case of θ, jackknifing over loci was used in calculating the standard deviation. The significance was determined by examining bootstrap generated 95% confidence interval around the mean. Finally, SAS (2004) was employed in performing multiple comparisons of polymorphic loci, F_{ST}, and heterozygosity, across the seasons with the non-parametric Kruskal-Wallis test (Daniel, 1990).

Results

Adult populations of *H. zea* were tested for temporal variation over a period of three years (2005-2007) (Table. 3.1). The adult moth collections made in Monroe Co., MS were subjected to molecular analysis using Inter Simple Sequence Repeats, ISSRs. The population collected in July of 2005 was utilized in determining the most suitable marker type for this study (see Chapter II). ISSR's were chosen to look into the relatedness and differences between the populations from three years. After efforts to collect emerging moths from diapause by using eclosion traps failed, we concentrated on differences in generations collected from early spring through the mid-summer and early fall. The adult

male moths collected from pheromone traps were analyzed using 8 ISSR primers. A total of 12 collections, four per year, based on seasonal occurrences, were examined in the study. Adult moth densities, based on the peak trap counts, were similar in all three years (Fig. 3.1). The adult moths were first captured in this area of Mississippi from late March to early April (Fig 3.1), and continued until early October in all three years. From the twelve populations tested against eight ISSR primers, 53 polymorphic loci were identified.

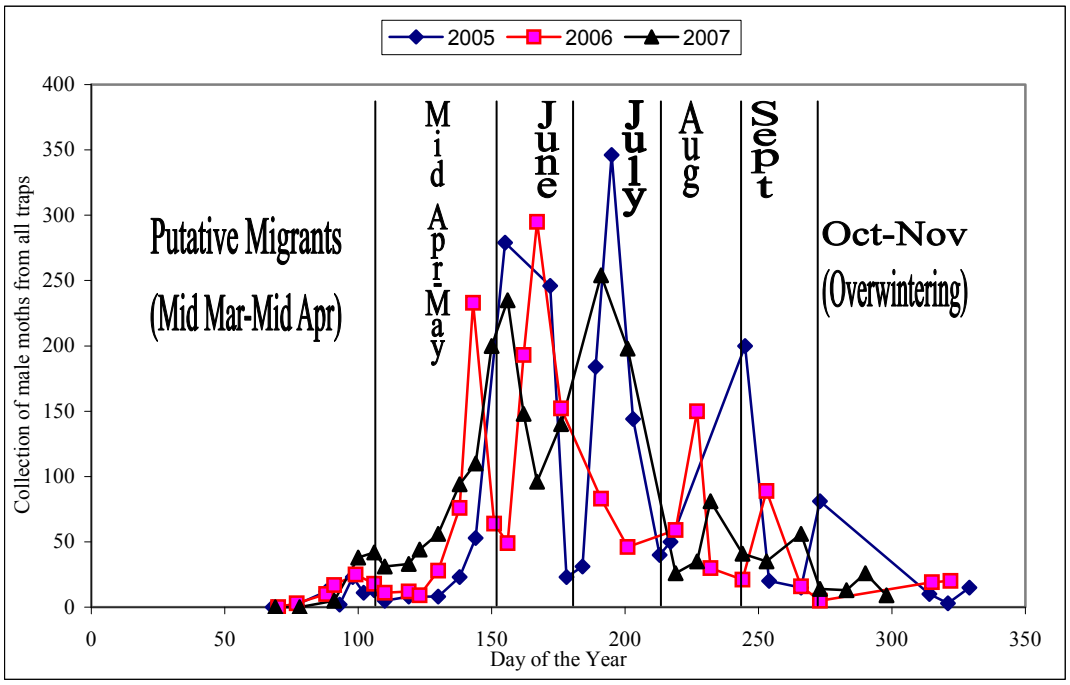

Figure 3.1 Adult male corn earworm population densities captured in pheromone traps in Monroe Co. Mississippi (2005-2007).

The amplified products for the given primers ranged from 200 to 3900bp in size. The number of bands studied per primer ranged from 3 to 11 with an average of 6 bands per primer tested. The markers showed differences among the corn earworm (CEW) moth

collections collected from three consecutive years. The eight ISSR primers tested showed different levels of heterozygosity among the 480 adults tested (Table 3.2). The percentage polymorphism for ISSR loci ranged between 22.2% and 42.7% with calculated heterozygosities ranging between 0.06 and 0.15 (Table 3.2).

Table 3.1 Corn earworm collections with corresponding sample sizes, collection dates and corresponding abbreviations.

Year	Month	Abbrev.	Sample size	Collection Timing
2005	March	M05	40	March-April
	June	J05	40	June
	July	L05	40	July
	August	A05	40	August
2006	March	M06	40	March-April
	June	J06	40	June
	July	L06	40	July
	August	A06	40	August
2007	March	M07	40	March-April
	June	J07	40	June
	July	L07	40	July
	August	A07	40	August

Table 3.2 ISSR primers used, Nei's unbiased heterozygosity (H_E) calculated for total *Helicoverpa zea* populations.

Primers	Sequence (5'-3')	No. of bands studied	H_E (SE)
816	CAC ACA CAC ACA CAC AT	8	0.15 (0.054)
818	CAC ACA CAC ACA CAC AG	7	0.08 (0.051)
825	ACA CAC ACA CAC ACA CT	11	0.09 (0.047)
826	ACA CAC ACA CAC ACA CC	6	0.08 (0.054)
827	ACA CAC ACA CAC ACA CG	8	0.13 (0.061)
828	TGT GTG TGT GTG TGT GA	6	0.14 (0.080)
850	GTG TGT GTG TGT GTG TYC	3	0.06 (0.047)
861	ACC ACC ACC ACC ACC ACC	4	0.13 (0.075)

Genetic diversity within the populations

A total of 53 polymorphic loci were employed in estimating the genetic diversity within the populations of CEW. The percentage of polymorphic loci (P) and corresponding Nei's (1978) unbiased mean heterozygosity (H_E) showed considerable variation among twelve collections (Table 3.3). P values ranged from 11.98% in samples from July of 2006 to 54.39% in case of the population from June of 2007. Similarly, the H_E values ranged from 0.033 to 0.203 in populations June of 2006 and 2007. The genetic diversity (P=54.39%; H_E=0.203) was highest in the 2007 midseason population and lowest (P=11.98%; H_E=0.041) in the 2006 midseason populations (Table 3.3).

Table 3.3 Sample sizes, percent polymorphism, and Nei's unbiased mean heterozygosity (H_E) per ISSR locus

Samples	Size	P	H_E (SE)
M05	40	21.91	0.11 (0.041)
M06	40	21.15	0.067 (0.046)
M07	40	18.35	0.066 (0.052)
J05	40	31.26	0.098 (0.068)
J06	40	8.85	0.033 (0.024)
J07	40	54.39	0.203 (0.082)
L05	40	29.48	0.108 (0.079)
L06	40	11.98	0.041 (0.023)
L07	40	45.53	0.173 (0.080)
A05	40	28.43	0.118 (0.085)
A06	40	33.60	0.097 (0.051)
A07	40	52.83	0.191 (0.079)

P: Percent polymorphic loci; H_E: Nei's unbiased mean heterozygosity.

Kruskal-Wallis tests (Daniel 1990) showed that year had a significant effect on heterozygosity (KW statistic=6.00; p=0.049). Multiple comparison statistics following Kruskal-Wallis showed that estimates of genetic diversity were higher in 2007 (H_E=0.182) than 2005 and 2006 (H_E=0.109, H_E=0.054, respectively).

Genetic differentiation among populations

The F_{ST} estimates from Weir and Cockerham's (1984) method were numerically higher than the Lynch and Milligan (1994) method, but statistically equivalent in differentiating populations. The pairwise LM F_{ST} values ranged from 0.024 (JA07) to 0.348 (MA05) (Table 3.4) (Figure 3.2). Adult moths early in the season were more distinct and differentiated from collections in 2 of 3 years (2005 and 2007). The pairwise F_{ST} estimates in these years were higher between the migrants and later generations (MJ05=0.341±0.231). Also comparisons between May and other collection dates were

significantly different from zero (Table 3.4). 2006 differed from the other two years as all successive generations were significantly differentiated from each other.

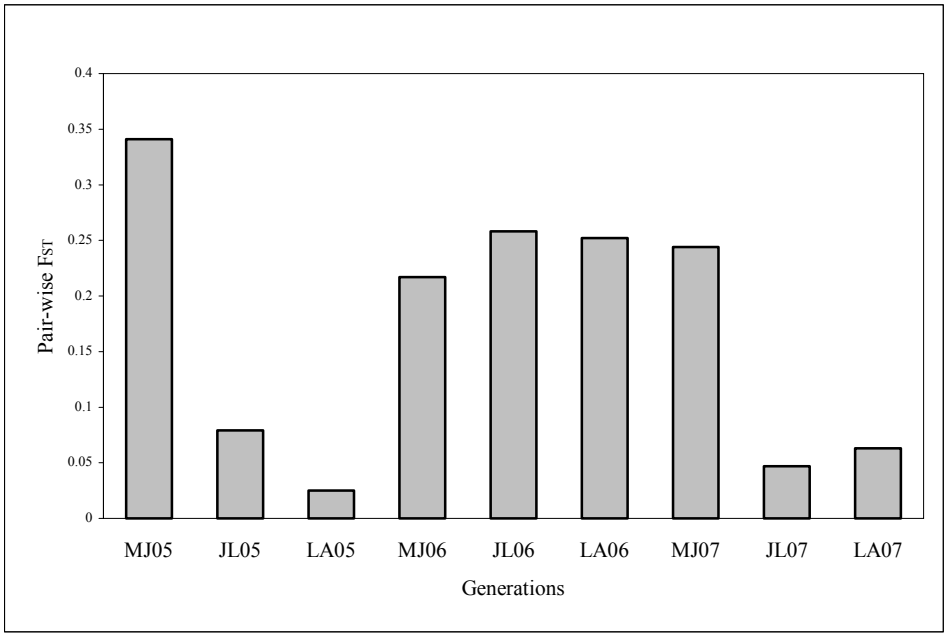

Figure 3.2 L&M Pair-wise F_{ST} values between the populations from different collections made from Monroe Co. MS.

Table 3.4 Pairwise F-statistics of corn earworm over generations using ISSR markers

Collection Time	Wright's F_{ST}	Weir's Theta (θ)	Lynch & Milligan F_{ST}
MJ05	0.133±0.218	0.350±0.244*	0.341±0.231*
ML05	0.147±0.169	0.349±0.218*	0.332±0.195*
MA05	0.155±0.170	0.367±0.224*	0.348±0.197*
JL05	0.048±0.084	0.084±0.140	0.079±0.114
JA05	0.065±0.090	0.121±0.148	0.115±0.117
LA05	0.025±0.065	0.032±0.106	0.025±0.08
AM56	0.093±0.115*	0.178±0.173*	0.163±0.139*
AJ56	0.157±0.134*	0.313±0.197*	0.269±0.159*
MJ06	0.109±0.162	0.239±0.217*	0.217±0.190*
ML06	0.106±0.152	0.235±0.205*	0.221±0.177*
MA06	0.109±0.124	0.276±0.180*	0.261±0.153*
JL06	0.114±0.128	0.297±0.178*	0.258±0.154*
JA06	0.114±0.128	0.297±0.178*	0.258±0.154*
LA06	0.108±0.124	0.280±0.172*	0.252±0.148*
AM67	0.093±0.108*	0.214±0.158*	0.202±0.129*
AJ67	0.113±0.110*	0.236±0.160*	0.219±0.138*
MJ07	0.153±0.140*	0.280±0.191*	0.244±0.172*
ML07	0.153±0.135*	0.272±0.187*	0.241±0.160*
MA07	0.131±0.121*	0.247±0.170*	0.216±0.146*
JL07	0.035±0.066	0.054±0.109	0.047±0.098
JA07	0.020±0.035	0.030±0.065	0.024±0.059
LA07	0.042±0.071	0.069±0.118	0.063±0.108

* Indicates 95% CI around mean does not include zero (significant)

Discussion

In an effort to understand genetic variation among the *H. zea* collections from three consecutive years, we tested them with newly developed molecular genetic markers that were promising and easy to handle. ISSR markers were particularly suited to our application. Eight random primers revealed 53 polymorphic loci that were effective in differentiating the populations. The genetic composition of the adult moths varied throughout the study period. The population densities were similar each year with four seasonal peaks from April-May till October. Corn and soybeans dominated the study area in all three years. Collections were made each year starting from early spring until the end of the season.

Heterozygosity levels and percentage of polymorphism showed variation among the twelve collections tested. Population differentiation declined in mid-summer in 2 of 3 years, especially in June and July of 2005 and 2007. These estimates support the concept of early spring movement as well as in-season movement between cropping areas. The early spring putative migrants were distinct from both early as well as later locally emerging generations. Overwintering populations that emerged the following season were distinct from the previous late season moths (June vs. previous fall). In other words, population differentiation estimates between August adults of 2005 (that produce overwintering pupae) and first generation, moths from first peak, of locally emerged adults (June 2006) (similarly between moths from late 2006 and early 2007) indicated relatively high levels of genetic differentiation, with higher values of F_{ST}.

Variations in population diversity were similar in pattern to a previous study, done with allozymes and RAPD markers (Han and Caprio 2002). The F_{ST} values showed a decline as the season proceeded, indicating the populations were genetically changing slowly. The adult moths that putatively migrate into the region from a different source, mainly from south, remain distinct due to the absence of local populations in the early spring. With the emergence of the local moths, migrants and the local adults mix leading to heterozygosity (Stadelbacher et al. 1986). This is evident from decreasing F_{ST} values as the season progresses. The pair-wise F_{ST} estimates between subsequent generations were always high between May and June collections (MJ05=0.341±0.231), but were low between the later generations (LA05=0.025±0.080), except in the year 2006 during which the trend was different and population genetic composition varied throughout the summer. The early spring moths were distinct from the rest of the populations (F_{ST} for MJ05=0.341±0.231) (Schneider 1999; Han and Caprio 2004). Usually, the dispersal rates of this pest are expected to be high during early part of the year. By mid-summer, wild hosts become less favorable and adults are attracted to silking corn. Adults start feeding on corn and dispersal rates decline with the availability of sufficient food. Previous studies showed that the expected heterozygosity was around 0.11 and 0.10 (Mallet et al. 1993 and Han and Caprio 2002, respectively). In our case, the expected heterozygosity ranged from 0.03 to 0.20, with an average of 0.11. This shows that the genetic diversity is low and stable and has not changed over time. These results can be at least partially attributed to the migratory and polyphagous nature of this pest.

Data from our study on migration and the contribution of early spring migrants to local populations indicated putative migrants to be very distinct from locally emerged adults. This trend was seen in 2 of 3 years of the study period. Due to the failure of our efforts in collecting overwintering adults, we were not able to make direct comparisons between putative migrants and overwintering populations. This study indicates that local populations were highly differentiated from early spring migrants. F_{ST}'s shows that the local emerged populations from one year were different from the next year's locally emerged populations. The comparison between late season adults (August) with the next season's locally emerged individuals (June-August) showed numerically higher levels of differentiation when compared to the in-season moths. Results from 2005, 2006 and 2007 provide evidence of northward migration of *H. zea* early in the season.

One hypothesis, that the overwintering survival favors different genotypes than the in-season survival could explain why late season collections are distinct from the next years emerging adult moths. Another hypothesis to explain why adult moths emerging in 2006 and 2007 differed from previous years collections of 2005 and 2006, respectively is that a portion of late season moths that overwinter have a different origin, possibly from north. Recent evidence from carbon isotope studies on host use by *H. zea* showed that late season moths from Texas fed on C4 plants (not cotton), while the local cropping area was occupied with C3 plants (cotton). This indicated a possible southward migration later in the season (Rennie 2003). Even though we have not looked into the C3 and C4 sources, our data does support this possibility. This hypothesis, which needs further investigation, explains why locally emerging moths might differ from previous years

(August) moths. Looking into the moths that we collected at the end of the season (mid-September) might help in getting supportive evidence.

Future studies comparing the moths from southern Mississippi with early spring putative migrants may help confirm the migration pattern. Also, investigating the last collections of the season will help in understanding if they have originated from north and a possible reverse migration. At the same time, continuous monitoring for genetic variations should be done to have a deeper insight.

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CHAPTER IV

CONCLUSION

Population genetics deals with the distribution and changes in the allele frequencies under the influence of evolutionary forces such as genetic drift, mutation, natural selection, and gene flow. We looked into some of these aspects in a major pest in the present agricultural world. *Helicoverpa zea*, commonly referred to as corn earworm, cotton bollworm, and tomato fruit worm, was tested for temporal variations in populations over three years. For this study, we have tested different molecular genetic markers for their applicability to the population genetic studies of *H. zea*. Three markers namely, microsatellites (SSR), inter simple sequence repeats (ISSR), and sequence-related amplified polymorphism (SRAP), were tested for the levels of polymorphisms and polymorphic information content. Forty individual moths collected from North Mississippi, Monroe Co. (July 2005) were tested against seven primer or primer combinations from each marker system.

The study has shown that the dominant markers, ISSRs and SRAPs, are more polymorphic and provided more information than SSRs. Microsatellites yielded twelve alleles, with 1.3 polymorphic products per primer. The polymorphic information content (PIC) was very low (0.21), when compared to the dominant markers, 0.34 and 0.30 in ISSRs and SRAPs, respectively. The high polymorphism in case of ISSRs and

SRAPs with 5.0 and 5.85 polymorphic products per primer indicated the applicability of these marker systems for examination of *H. zea* population genetics. Based on the polymorphic information content and the ease of use in procedure and analysis, we used ISSRs to study the temporal variations in *H. zea*. All the data were handled carefully by testing for reproducibility as well as implementing corrections for dominant markers. We concluded that any of the dominant markers could be used for studies with *H. zea*. As far as is known, this was the first application of SRAP on insects.

Using eight ISSRs, we looked into the temporal variations among four collections per year over three years among populations collected from Monroe Co. in northern Mississippi. The population density patterns were consistent in all three years, with early spring populations starting around mid-March. Populations were less differentiated as the season progressed in 2 of 3 years. Polymorphism and heterozygosity levels varied among the twelve populations tested. Genetic diversity was stable over generations even though there was variation within each generation. A measure of population differentiation, the fixation index, F_{ST} was higher between the generations in the beginning of the season and then reduced as the season progressed. This study has indicated a similar trend in genetic diversity estimates as obtained in earlier studies (Mallet et al. 1993; Han and Caprio 2002). Average heterozygosity levels of 0.11 from our data coincided with data from 1993 and 2002. The polyphagous nature and migratory ability of this pest explains these patterns and changes. Large-scale migration coupled with breeding success results in lower genetic differentiation.

The transgenic crops and the refuge structure currently implemented, including the widely available natural wild host refuge, are probably efficient in maintaining the

resistant allele frequencies at levels where field control will not be compromised. With frequent movement of *H. zea* adults between fields and mixing among generations, the risk of field resistance is low. Our data showed that there is variation in how much putative migrants are contributing to the local gene pool. Adult moths caught from June local populations were distinct from the early putative spring migrants and this trend was seen in 2005, 2006 and 2007. The data are consistent with the hypothesis that a substantial proportion of the June population is derived from locally overwintering moths in 2005 and 2007. Our data differed in 2006 with different trend in genetic variation. Given the contribution of locally overwintering moths, it seems prudent to continue the use of refuges (local resistance management) in the northern half of Mississippi.

Our data showed that there is more need for sampling populations over years for these patterns of variation. We need extended monitoring of populations to see if the pattern we observed are common. If that's the case then, those variations will have implications on resistance management as far as the refuges are concerned. As these refuges are based on the concept of mixing of moths from movement between transgenic and non-transgenic crops, variations in local and non-local adult densities will have a strong impact on the resistance management success. From our data, there was less contribution from migrants to the local population, indicating the continued need for refuges in this area of the country, but requires more supportive evidence as the 2006 data showed different pattern. Further studies comparing populations from different geographic regions are needed to gain more insight into the resistance management in Bt crops in Mississippi.