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Population structure of the pestiferous moth *Helicoverpa armigera* in the Eastern Mediterranean using RAPD analysis

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The genetic structure of the cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), was studied in the eastern Mediterranean. Moths were sampled in six locations (five in Israel, and one in Turkey) and their genetic relationship was analysed using RAPD-PCR. Three 10-oligonucleotide primers revealed 84 presumptive polymorphic loci that were used to estimate population structure. Results reveal low level of genetic distances among Israeli and Turkish populations. The estimated values of F_{ST} and θ for the eastern Mediterranean populations were very low across all populations, indicating a high level of gene flow. Four distinct RAPD-product profile types were defined, and found in all Israeli and Turkish populations. Although no isolation by geographical distance was detected, topographical barriers may play a role in such isolation.

Keywords: gene flow, *Helicoverpa armigera*, migration, population genetics, RAPD.

Introduction

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is one of the most widely distributed agricultural pests throughout Africa, the Middle East, southern Europe, India, central and south-eastern Asia, eastern and northern Australia, New Zealand, and many eastern Pacific Islands (Fitt, 1989). Its distribution is roughly within the latitude boundaries of 45°N and 45°S, excluding the Americas (Hardwick, 1965). Its polyphagous characteristics, high mobility and ability to develop resistance to most common insecticides have enabled it to become an important pest over a wide geographical range.

The Israeli populations of *H. armigera* undergo a short day pupal diapause and are suspected to migrate in the eastern Mediterranean region (Zhou *et al*., 2000a,b). In insect populations of little mobility, the length of critical photoperiod that induces diapause is indicative of geographical latitude: the further the insect is from the equator, the longer the critical day length for diapause induction (Danilevskii, 1961). In *H. armigera*, however, this pattern of critical threshold for diapause induction does not seem to hold over a wide geographical range between latitudes 25°S and 44°N (Hmimina *et al*., 1993). A high level of gene flow may be hampering spatial differentiation in diapause induction.

Two classes of methods can be used to estimate the extent of gene flow in natural populations. 'Direct methods' measure dispersal distance and breeding success of dispersers to infer about gene flow level at the time of observation. 'Indirect methods' rely on allele frequencies, DNA sequences or restriction fragments, to estimate the historical levels of gene flow that are likely to produce the observed genetic patterns (see Slatkin, 1987 for general discussion).

In the case of *H. armigera*, only a few allozyme markers have been used to analyse population genetic structure in different regions (Daly & Gregg, 1985; Nibouche *et al*., 1998). Its wide geographical distribution and suspected high dispersability call for a greater genetic understanding of its population structure.

A diverse array of molecular techniques is available for high-resolution genetic studies of population level processes. Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR), using a single primer, simultaneously amplifies many regions of genomic DNA (Williams *et al*., 1990). By using several different primers, polymorphisms at many RAPD loci

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may be detected among individuals. RAPD-PCR analysis can therefore potentially increase the resolution of genetic differences among individuals in population genetic studies. This method is used widely in the determination of population genetic structure, because it has the particular advantage of being the least technically demanding, and no prior knowledge of DNA sequence is required. In the present study, we used 84 RAPD loci, detected by 3 primers, to analyse the genetic structure in 6 eastern Mediterranean *H. armigera* populations.

Materials and methods

Adult male moths of the cotton bollworm

H. armigera adult males were field-collected using sex pheromone traps during May–June 1997, in five locations in Israel (three along the rift valley in the east: Hula valley, Beit She'an and Hatzeva; and two along the coastal plain in the west: Kfar Menahem, and Nachsholim; Table 1). Male moths were also field-collected during August 1997 in Adana, Turkey. Live moths were placed in absolute ethanol, transferred to the laboratory and stored at −20°C. DNA preparation was executed within three weeks.

Preparation of cotton bollworm DNA

As female adults may accept sperm from males, only adult *H. armigera* males were used for DNA preparation. The method is modified from Garner & Slavicek (1996). Each moth was taken out of the ethanol, dried well, and its legs and wings were removed. The moths were homogenized individually using a mortar and pestle in 380 μL grinding buffer containing 10 mM Tris-Cl (pH 7.5), 60 mM NaCl and 10 mM EDTA, and 320 μL were finally transferred to a 1.5-mL tube. An equal volume of postgrinding buffer consisting of 200 mM Tris-Cl (pH 9.0), 30 mM EDTA and 2% SDS was added to the homogenate, and treated with 128 μg Proteinase K at 50°C overnight. Sodium acetate (pH 4.8) was added to a final concentration of 0.3 M. The solution was first extracted with an equal volume of buffered phenol, and then extracted with an equal volume of chloroform/isoamyl alcohol (24/1). Two volumes of ethanol were added to precipitate the DNA. After no less than 2 h at −20°C, the DNA was pelleted by centrifugation, and air-dried at 37°C for 2 h.

The DNA pellet was resuspended in 150 μL double-distilled water and treated with 30 μg RNAse A for 2 h at 37°C. The DNA was then extracted again with phenol and chloroform and precipitated with ethanol as above. The DNA pellet was resuspended in 100 μL double-distilled water and stored at 4°C for future use.

Table 1 Sample location, size and date for *Helicoverpa armigera* collections in the eastern Mediterranean region

Location	Sample size	Longitude	Latitude	Date
Adana, Turkey	35	30°20′E	37°00′N	08/1997
Hula valley	30	35°40′E	33°10′N	06/1997
Nachsholim	30	34°55′E	32°45′N	06/1997
Beit She'an	34	35°30′E	32°30′N	06/1997
Kfar Menahem	44	34°50′E	31°50′N	04/1997
Hatzeva	20	35°15′E	30°45′N	05/1997

RAPD-PCR method

RAPD-PCR amplifications were performed using a modification of the method of Garner & Slavicek (1996). Each of the 25 μL reactions contains 20 ng DNA, 1.5 mM $MgCl_2$, 0.1 M each dNTP, 0.2 μM primer, 0.37 unit *Taq* DNA polymerase (MBI). Mixtures were covered with a drop of mineral oil. The samples were incubated in 0.5 mL tubes in an MJ Research thermal cycler for 2 min at 94°C and then for 45 cycles consisting of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min. A negative control (without template DNA) was included in each set of reactions. Half volumes of reaction products together with DNA size markers were electrophoresed in 1.5% agarose gels using Tris-Boric Acid-EDTA buffer followed by ethidium bromide staining of the DNA and visualization under UV light. The same band pattern was obtained when the reaction was repeated with the same DNA sample and the same primer.

Fifty-five 10-oligonucliotide primers were initially screened using pooled samples of DNA from each population. Equal amounts of DNA from 10 individual moths within each population were pooled together. DNA extracted from a Chinese *H. armigera* population (laboratory colony supplied by the Shanghai Institute of Entomology, Chinese Academy of Science) was used as control. A primer was regarded useful if it generated consistent and reproducible polymorphic markers. Three primers were selected as potentially useful: OPD-02 (5′-GGACCCAACC-3′) and OPG-06 (5′-GTGCCTAACC-3′) from Operon Technologies, and 102 (5′-GGTGGGGACT-3′) from the University of British Columbia.

The bands on gels were scored using the Gel-Pro Analyser program (Media Cybernetics, L.P.) in a sensitivity of molecular weight ±0.8%. Only unambiguous bands of between 400pb to 1300pb were scored. The RAPD-PCR database was established as the presence (1) or absence (0) of every polymorphic band for all of the individual samples.

Data analysis

Unlike allozymes, RAPD products are dominant, not codominant. With dominant markers, heterozygotes are indistinguishable from homozygotes, and allelic information is therefore not directly available from RAPD products. RAPD-PCR polymorphisms were analysed as alleles and the frequency of alleles was estimated, based on the following assumptions: (i) RAPD products segregate as dominant alleles in a Mendelian fashion; (ii) genotype frequencies at RAPD loci are in Hardy–Weinberg proportions; (iii) alleles in homozygous recessive individuals are identical in state *(iis)* (i.e. they arose from identical mutations) among and within individuals; (iv) dominant, amplified alleles are similarly *iis* (Apostol *et al*., 1996).

The RAPD-PCR database was analysed using the RAPDFST program (Black, 1995). In this program, F_{ST} was estimated according to the methods of Wright (1951), Weir & Cockerham (1984), and Lynch & Milligan (1994). The significance of F_{ST} was tested using the nonparametric permutation approach described by Excoffier *et al*. (1992). Because of the inability to distinguish heterozygotes from RAPD data, it is not possible to calculate F_{IS}, which indicates the average of deviations within the subpopulations.

We used the relationship of $F_{ST} \approx 1/(4Nm + 1)$ to estimate the number of migrants per generation among subpopulations (Nm) (Wright, 1951). A value of $Nm > 1$ is considered sufficient to make gene flow overcome genetic drift (Wright, 1931). While it has been suggested that the estimation of gene flow based on F_{ST} methods is preferable to other methods that use allele frequency data, such as the private alleles and maximum likelihood methods (Slatkin & Barton, 1989; Coll *et al*., 1994), there have been several recent criticisms on its unrealistic assumptions (e.g. Whitlock & McCauley, 1999).

The data obtained were also analysed by cluster analysis. Nei's Distance (Nei, 1972) with Lynch & Milligan's (1994) correction, were used to compute genetic distances among subpopulations using the RAPDDIST program (Black, 1995). A Mantel test (Mantel, 1967) was used to examine the relationship between genetic and geographical distances. The similarity of RAPD profiles between each possible pair of individuals from all populations was calculated using Nei & Li's (1979) similarity index, based on the degree of sharing common bands measured using RAPDPLOT (Black, 1995). Using the similarity index matrices, a dendrogram was produced using the neighbor joining method (NEIGHBOR program in PHYLIP 3.5C). All individuals were grouped into several distinct RAPD product profiles based on the clustering seen. A chi-square test was performed to compare the distribution of these RAPD types among populations.

Results

Genetic distances among populations

Overall, 84 out of 88 amplified DNA fragments generated by three primers were found to be polymorphic at the 99% level. Allele frequencies were estimated from the frequency of homozygous recessive individuals. Across all studied RAPD loci, the mean expected heterozygosity was 0.1013 ± 0.0683 (SD).

The genetic distances among various geographical populations of *H. armigera* are presented in Table 2. The mean genetic distance among Israeli and Turkish populations, was 0.0029 ± 0.00079 (SD). No significant correlation was detected between genetic distance and geographical distance among the studied populations (Mantel test, $P > 0.05$). Effects of geographical distance in separating populations may be overcome by the moth's high mobility. However, unlike geographical distance, it appears that topographic barriers have an important effect on genetic structure of *H. armigera* populations (see results in next section).

Genetic similarity among individuals

Based on the similarity in RAPD-product profiles, all individuals were grouped into four distinct RAPD-product profiles. Each group was assumed to be a RAPD product pattern type, and individuals belong to an identity RAPD type were thought to be genetically similar. The frequencies of the four RAPD types in each population are shown in Fig. 1. All sampled populations contain these four type-individuals. The populations were divided into two patterns based on their RAPD-product type. The pattern of the Israeli populations collected east of a North-to-South mountain range (altitude 600–1200 m) (Hula valley, Beit She'an, and

Table 2 Nei's genetic distances between pair-wise populations of *Helicoverpa armigera*

Populations	1	2	3	4	5	6
1 Kfar Menahem	0					
2 Beit She'an	0.0041	0				
3 Nachsholim	0.0023	0.0028	0			
4 Hula valley	0.0030	0.0019	0.0017	0		
5 Hatzeva	0.0045	0.0031	0.0035	0.0025	0	
6 Turkey	0.0041	0.0026	0.0031	0.0023	0.0032	0

Hatzeva), together with the Turkish population, differ significantly (χ^2-test, $P < 0.001$) from the western Israeli populations (Nachsholim and Kfar Menahem; Fig. 1).

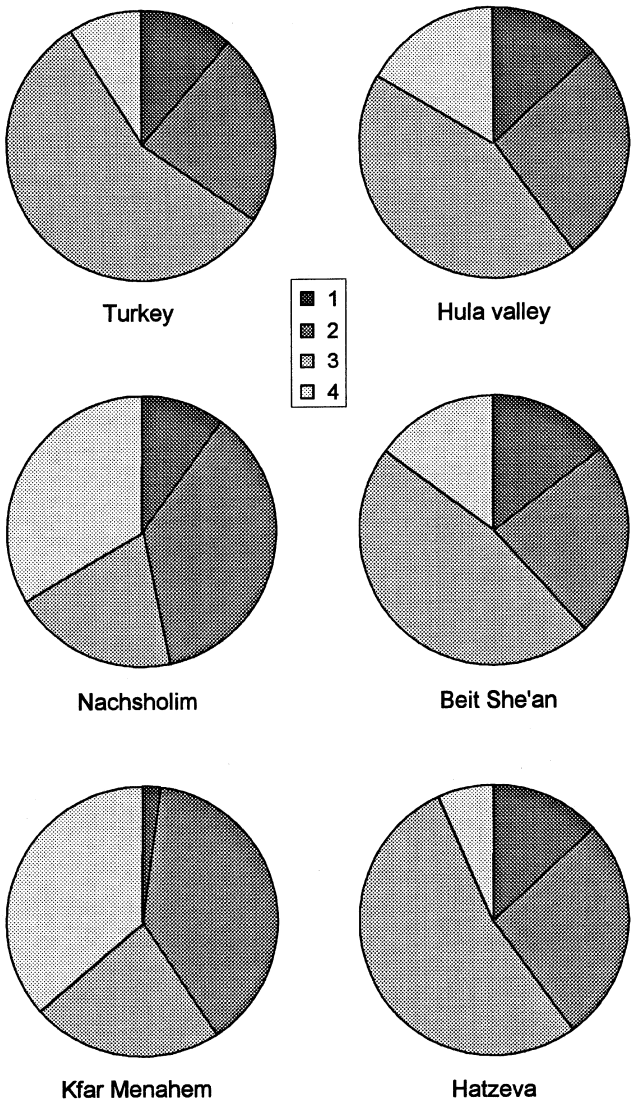

Fig. 1 Pie-diagrams of the proportion of RAPD-produced pattern types in each sampled *Helicoverpa armigera* population. Four RAPD types are presented.

Estimation of F_{ST} *and Nm*

Both RAPD profile and genetic distances among *H. armigera* populations suggest a weak differentiation between populations found west and east of the Israeli mountain range. Weir & Cockerham's (1984) θ, Lynch & Milligan's (1994) F_{ST} and Wright's (1951) F_{ST} were estimated for each locus at the following geographical levels: (i) all Israeli and Turkish populations; (ii) the western Israeli populations; and (iii) the eastern Israeli and Turkish populations. The number of effective migrants, *Nm*, was estimated from these *F*-statistics. The F_{ST} and θ averaged over all loci, and *Nm* values are shown in Table 3. While the estimates of Weir & Cockerham's θ and Lynch & Milligan's F_{ST} were similar to each other, Wright's F_{ST} seems to give lower estimates and Lynch & Milligan's F_{ST} tends to generate large standard deviation of estimates by giving more extreme estimates than the others.

For all populations from Israel and Turkey, F_{ST} and θ estimates were very low, but significant (Table 3). However, lower levels of *F*-statistics are detected, as the geographical scale of *H. armigera* populations becomes smaller (Table 3). For example, Weir & Cockerham's θ value decreases from 0.011 ($P < 0.05$) for overall population to 0.004 and 0.009 (both $P > 0.05$) for the regional levels of eastern Israeli and Turkish populations, and western Israeli populations, respectively. Since θ and F_{ST} values estimated at these smaller scales are not significantly greater than zero, there would be no upper bound for the estimated number of migrants. A higher *Nm* value estimated within regions on either side of the mountain range, rather than between these regions, indicates that gene flow may be limited to some extent by the mountain range.

Discussion

Our study of the genetic structure of *H. armigera* populations in the eastern Mediterranean region, based on RAPD markers, indicates the occurrence of a high level of gene flow in this area. It seems that geographical distance by itself is insufficient for isolating adjacent

Table 3 Estimates of F_{ST} or θ ($\pm$SD), and *Nm* at different regional levels of *Helicoverpa armigera* population

	Weir & Cockerham's		Lynch & Milligan's		Wright's	
Region level	θ	*Nm*	F_{ST}	*Nm*	F_{ST}	*Nm*
Turkish & Israeli populations	0.011* (0.0019)	21.7	0.013 (0.0034)	19.1	0.020* (0.0016)	12.1
Turkish & Eastern Israeli & populations	0.004 (0.0018)	62.4	0.004 (0.0032)	66.9	0.016 (0.0013)	15.2
Western Israeli populations	0.009 (0.0026)	26.8	0.008 (0.0020)	30.1	0.010 (0.0013)	25.7

*F_{ST} or θ values are significantly greater than zero ($P < 0.05$).

populations, but natural barriers may play a role in such isolation. We found that *H. armigera* individuals in all studied populations can be grouped into four RAPD types, based on their RAPD profiles. The mixture of individuals representing all four RAPD types suggests frequent movements over this geographical range.

A related study (Zhou *et al*., 2000a) suggests that *H. armigera* moths disperse from east Africa to Israel during early spring. To test this possibility, a preliminary RAPD analysis was conducted using a small number of moths from Cairo, Egypt and Melkasa, Ethiopia. It appears that the differentiation among the eastern Mediterranean and east Africa is still very low ($\theta = 0.015$) and gene flow is high ($Nm = 16.2$), thus providing further support to the notion that a large number of individuals move across this region.

Using allozyme electrophoresis, Daly & Gregg (1985) found a mean heterozygosity of 0.113 in Australia *H. armigera* populations, whereas Nibouche *et al*. (1998) found a mean heterozygosity of 0.104 in African and European populations. Both results are similar to the value of 0.101 in the present study, although it appears that overestimation of the level of heterozygosity may have resulted from the selective use of oligonucleotide primers that produce polymorphic markers. A mean genetic distance of *H. armigera* in Australia was estimated as 0.004 ± 0.002 (SD) (Daly & Gregg, 1985), which is higher, although not significantly so, than the distance found in this study [0.0029 ± 0.00079 (SD)]. A θ-value of 0.007 and mean genetic distance of 0.002 were estimated across a 4000-km range along the western coast of Europe and Africa, (Nibouche *et al*., 1998). Both values are lower than the values obtained in our study.

The disadvantage of using RAPD polymorphisms to study population genetic structure is that the majority of alleles segregate as dominant markers (Williams *et al*., 1990). The main difference between codominant molecular markers, such as RFLPs or DNA sequences, and dominant RAPD markers, lies in the lack of genotypic information for the assayed individuals. To estimate allele frequency, one has to make several assumptions. These additional assumptions allow the use of the highly polymorphic RAPD markers, but at the cost of decreased precision compared to that of codominant markers (Lynch & Milligan, 1994).

Because of its pest status in agriculture, *H. armigera* populations are often exposed to severe selection pressure imposed by insecticide treatments (Fitt, 1989). High levels of gene flow may retard the evolution of insecticide resistance, because local populations are transfused with sensitive individuals. In contrast, high levels of gene flow may promote the evolution of resistance when resistant individuals move into susceptible populations and enjoy relatively high fitness under insecticide selective pressure (Slatkin, 1987). High mobility is also a desirable trait for an insect pest because its populations will undergo frequent cycles of extinction and recolonization in many agroecosystems. Thus, the establishment of new local populations is an effective way for spreading new adaptations (Wade, 1980).

The high level of gene flow in *H. armigera* in the eastern Mediterranean region, and probably east Africa, suggests that a frequent movement of a large number of individuals occurs in this area. However, either large-scale, long distance migration or trivial displacement with successful breeding could result in low genetic differentiation. Several previous studies have suggested that *H. armigera* exhibits migratory behaviour. Pedgley (1985) showed that *H. armigera* migrated up to 1000 km to reach Britain and other parts of Europe from sources in southern Europe and North Africa. In Australia, Farrow (1984) observed *H. armigera* in light traps on Willis Island in the Coral Sea, 450 km off the coast of Queensland. Gregg *et al*. (1993) caught *H. armigera* using a tower-mounted light trap at an altitude of 1560 m at Point Lookout on the coast of Queensland, where the moths' host plants were very scarce and local breeding was considered impossible. In India, *H. armigera* collected in light traps during March–April, showed cessation of ovarian development, accumulation of fat body reserves and an increase in wing span and body length. These parameters suggest a migration-related morphological phase that had been expressed during the migratory season (Vaishampayan & Singh, 1996).

Results of a related study on physiological descriptors and pheromone trapping data (Zhou *et al*., 2000a,b) provide further support for long-distance spring migration of *H. armigera* in the eastern Mediterranean region. It is not clear, however, whether such migration is followed by a return, North-to-South, flight in autumn.

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