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Molecular Characterization of *Helicoverpa armigera* (Hubner) Populations from Punjab, India

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ABSTRACT

Helicoverpa armigera Hubner (Lepidoptera: Noctuidae) is a highly polyphagous and migratory pest of several economically important crops in parts of Africa, Asia, Australia and Europe. Molecular variability of *H. armigera* infesting different crops viz. non-Bt cotton, pigeon pea, Egyptian clover, sunflower and tomato mostly from the areas near Ludhiana, Punjab, India and inter-location variability on Egyptian clover across three agroclimatic zones of Punjab was studied by mitochondrial cytochrome oxidase I sequence alignments. Based on sequence analysis of mitochondrial cytochrome oxidase I gene, the Ludhiana population differed from Gurdaspur population by the 0.2% and from Abohar population by 0.5% while the latter two differed from each other by 0.7%. Among populations infesting various host plants, cotton population differed from sunflower by 0.2%, Egyptian clover by 0.3% and tomato and pigeon pea by 0.4% suggesting little genetic variation among populations collected from different regions of Punjab and infesting various hosts.

Key words: Molecular characterization, *Helicoverpa armigera*

Introduction

Helicoverpa/Heliothis species (Noctuidae) constitute a worldwide pest complex of great economic importance. Several of these species are highly polyphagous, feeding on a wide range of crops (Cunningham and Zalucki, 2014; Sosa-Gomez *et al.*, 2016). Three *Helicoverpa/Heliothis* species occur in India, viz. *Helicoverpa armigera* (Hubner), *Helicoverpa assulta* (Guenee) and *Heliothis peltigera* (Denis & Schiffermuller). Eleven years light trapping data from ICRISAT centre, Patancheru (Andhra Pradesh) for these species revealed that they constituted 99.2, 0.6 and 0.2 per cent of the total catch, respectively (Pawar *et al.*, 1986a,b). The cosmopolitan species *H. armigera* is a highly polyphagous pest of many economically significant crops in parts of Africa, Asia, Australia and Europe (King, 1994). The adult stages are highly mobile and capable of migration (Feng *et*

al., 2009), allowing adaptation to a changing mosaic of hosts in ephemeral environments. Worldwide the annual control costs and production losses due to *H. armigera* amounted to US\$5 billion (Anonymous, 2005), while in India damage worth US \$ 1 billion has been estimated (Subramanian and Mohankumar, 2006). In India this insect occurs as a major pest in many economically important crops, including cotton, pigeon pea, chickpea, Egyptian clover, tomato, okra, and blackgram etc. *H. armigera* was the predominant species in Punjab causing economic damage to many *Kharif* and *Rabi* crops, while *H. peltigera* was confined to castor and sunflower crops (Singh and Sidhu, 1990).

The genetic plasticity of *H. armigera* is apparent in its ability to detoxify many synthetic insecticides in addition to the secondary plant metabolites present in its wide array of host plant species. Differences do exist in the behaviour of *H. armigera* populations in-

festing various host plants and geographically distinct groups to the extent that the existence of at least subspecific differences between populations cannot be completely ruled out (Reed and Pawar, 1982). With the advent of molecular markers, a new generation of markers has been introduced over the last two decades, which have revolutionized the entire scenario of biological sciences. Genetic (electrophoretic) variation was examined in the two species of *Helicoverpa/Heliothis* found in Australia, *H. armigera* (Hubner) and *H. punctigera* Wallengren. They could be differentiated by seven loci, which enabled identification of individual eggs and small larvae not normally distinguishable by their morphology. The genetic distances between the two species were 0.34 ± 0.02 . The percentage of loci polymorphic in the species, 32 per cent and the mean heterozygosities 11.3 per cent for *H. armigera* and 10.8 per cent for *H. punctigera* are lower than those reported in the American species, *H. virescens* (F.) and *H. zea* (Boddie). Populations throughout Australia were differentiated from each other, but there did not appear to be marked geographical pattern to the variation. Genetic distances between populations of *H. armigera* were low (<0.01). It was concluded that the effective population size of *Helicoverpa/Heliothis* was large and that significant gene flow probably occurred between widely separated regions (Daly and Gregg, 1985). Understanding the exact nature of the pattern of genetic diversity and inter-relatedness between populations would help to exactly pin down the causes for frequent outbreaks of the pest populations over ecosystems.

Due to the wide distribution and migration ability of *H. armigera*, reliable molecular genetic markers that enable population comparisons within and between countries are required. Furthermore, such markers would be even more useful if they could be applied to related pest species within the genus (i.e. *H. zea*, *H. assulta* and *H. punctigera*). Different levels of genetic differences among geographic populations of *H. armigera* have been shown using isozyme (Daly and Gregg, 1985), allozyme (Nibouche *et al.*, 1998), RAPD (Yenagi *et al.*, 2012; Basavanneppa *et al.*, 2013), SSR (Khaiban *et al.*, 2010), Microsatellite (Scott *et al.*, 2005), cadherin gene (Gujar *et al.*, 2007) and EPIC- PCR (Templeton, 1990). Differences based on genetic analyses indicate considerable subspecific level variations among *H. armigera* populations of India (Chakravarty *et al.*, 2021). The extent of genetic variation between geographical populations

depends on several factors, including gene flow between populations, host range and time since separation (Behere *et al.*, 2013). Partial (511 bp) mitochondrial DNA (mtDNA) Cytochrome Oxidase-I (COI) sequences were obtained for 249 individuals of *H. armigera* sampled from Australia, Burkina Faso, Uganda, China, India and Pakistan which were associated with various host plants. Single nucleotide polymorphisms (SNPs) within the partial COI gene differentiated *H. armigera* populations into 33 mtDNA haplotypes. Partial regions of the mitochondrial DNA (mtDNA), the cytochrome oxidase subunit I (COI) and the cytochrome *b* (*Cyt b*) genes were amplified by PCR and digested with restriction endonucleases. The restriction patterns, generated by the endonucleases *Bst*Z17I and *Hph*I, demonstrated reliable differentiation of the four *Helicoverpa* pest species (*H. armigera*, *H. assulta*, *H. punctigera* and *H. zea*) (Behere *et al.*, 2008).

Understanding the genetic variation among the *Helicoverpa/Heliothis* spp. populations infesting different hosts and occurring in different geographical conditions has become essential to understand the variation in various biological, morphological, physiological characters of pest and also to predict their spatial and temporal occurrence, as well as to devise effective management strategies. A scan through literature revealed that most of studies on molecular diversity of *H. armigera* were undertaken in south Indian region and studies from northern region are lacking. Present studies were undertaken to understand molecular diversity of *H. armigera* populations infesting different hosts and occurring in different agroclimatic zones of Punjab.

Materials and Methods

Collection

In case of inter-host variability experiment, *H. armigera* larvae were collected from different crops (Table 1) namely, non-Bt cotton, pigeon pea, Egyptian clover (berseem), sunflower and tomato mostly from the areas near Ludhiana and in case of inter-location variability experiment, larvae were collected from Central plain zone (Ludhiana), Western zone (Abohar) and Sub mountain zone (Gurdaspur) infesting Egyptian clover (Table 2) and were maintained on their respective hosts until pupation in a plant growth chamber, after rearing for one generation ($25 \pm 2^\circ\text{C}$, temperature; $70 \pm 5\%$ Relative humid-

Table 1. Different crops of collection of *Helicoverpa armigera* populations

Crop	Scientific name	Season	Location
Egyptian clover (berseem)	<i>Trifolium alexandrinum</i>	Rabi	Ludhiana
Tomato	<i>Lycopersicon esculentum</i>	Rabi	Ludhiana
Sunflower	<i>Helianthus annuus</i>	Rabi	Ludhiana
Cotton	<i>Gossypium arboreum</i>	Kharij	Ludhiana
Pigeon pea	<i>Cajanus cajan</i>	Kharij	Ludhiana

ity) larvae were processed for experimentation.

DNA isolation from *Helicoverpa armigera* larvae:

The total genomic DNA was isolated from single representative larva from each population using a modified CTAB method (Cubero *et al.*, 1999). Two larvae from each location/host were used for study representing two replications. Larva was macerated with the help of micropestle in 200 µl of CTAB solution in 1.5 ml microcentrifuge tube. The tubes were incubated at 65°C for 60 min with intermittent mixing of tube contents. The lysed mass was extracted with 500 µl chloroform: isoamyl alcohol (24:1). The contents were mixed by vortexing to form an emulsion followed by centrifugation at 10000 rpm for 1 min. The DNA in the upper aqueous layer was transferred to a fresh 1.5 ml microcentrifuge tube. DNA was precipitated by adding 50 µl of 2.5M sodium acetate (pH 5.2) and 500 µl of isopropanol. The precipitated DNA was collected by centrifugation (10,000 rpm, 5 min) and the DNA pellet was washed with 70 per cent ethanol and allowed to air dry at room temperature. The dried DNA pellet was dissolved in 100 µl of TE buffer containing DNase free pancreatic RNase A (10µg per ml) and stored at -20°C until used. The quality of isolated DNA was determined by horizontal agarose (0.7 per cent containing ethidium bromide @1µg per ml) gel electrophoresis in 1x TAE buffer. The DNA bands were visualized and photographed under a UV transilluminator in 'Ultra Cam Gel Documentation system.

Phylogenetic analysis of mtCOI sequences of *H. armigera*

Amplification of mtCOI: The mtCOI gene from genomic DNA of larva was amplified using following lepidopteran mt COI specific primers (Hajibabaei *et*

al., 2006) (Forward: 5-ATTCAACCAATCA TAAAGATATTGG-3¢ and Reverse: 5¢-TAAACTTCTGGATGTCCAAAA ATCA-3¢).

PCR reaction were performed in 20 ml reaction, each containing 20ng template DNA solution (1ml), 1 mM dNTPs mix (5ml), Primers (1ml each), Taq polymerase 5 units/µl (0.4 ml), 15mM MgCl₂ in 10X Taq reaction buffer (2ml) and Sterile Milli-Q H₂O (9.6ml). All PCR- amplifications were accomplished in a programmable DNA thermocycler (Mastercycler Gradient- eppendorf™) using a PCR amplification programme consisting of 95°C for 5 min (preheating), 95°C for 1 min, 55°C for 1 min, 72°C for 2 min (30 cycles), 72°C for 10 min (final extension) and stored at 4°C until used. The PCR mixture was subjected to agarose gel (0.7 %) electrophoresis and amplification profile was visualized and photographed using UV-Gel Documentation system (UltraLum).

Cloning of mt CoI: The agarose block containing the specifically amplified DNA band was excised from the agarose gel with a clean, sharp scalpel blade and transferred to a 1.5 µl microcentrifuge tube. The DNA fragments were gel purified using 178 QIAquick Gel Extraction Kit' (Qiagen) as per manufacturer's protocol. The size and concentration of the eluted DNA was ascertained by electrophoresis on 0.7 per cent (w/v) agarose gel in comparison with a DNA size marker (100 bp DNA ladder plus).

The purified DNA fragments were cloned into a 'PCR cloning vector pTZ57R/T' and the ligation reaction product was transformed into *Escherichia coli* DH5-alpha host cells using 'InsTAclone™ PCR Cloning Kit' (Fermentas Life Sciences) using manufacturer's protocol. The competent cells with ligation mixture were uniformly spread plated on a

Table 2. Characteristics of areas of collection of *Helicoverpa armigera*.

Location	Agroclimatic zone	Latitude	Longitude	Altitude (feet)
Ludhiana	Central Plain zone	30.9000° N	75.8500° E	797
Gurdaspur	Sub-mountain undulating zone	32.0333° N	75.5167° E	793
Abohar	Western zone	30.1500° N	74.1833° E	590

LB-ampicillin- X-gal/IPTG- agar plate. The plates were incubated overnight at 37°C and observed for growth of blue (non-recombinant) and white (recombinant) coloured clones. Five individual recombinant (white) clones were picked up with a sterile tooth pick and inoculated into 3 ml of LB-ampicillin broth. The tubes were incubated overnight at 37°C under shaking conditions (180 rpm). Using this broth culture, miniprep plasmids were isolated using 'alkaline lysis method' of Birnboim and Doly (1979). Subsequently all plasmid DNA preparations were stored at -20°C, until used.

The size of recombinant plasmids was determined by horizontal agarose gel electrophoresis (0.7 %). 8µl of plasmid DNA mixed with 1.5µl of 6X 'gel loading buffer' was loaded in agarose wells and run in TAE buffer at constant voltage (75 V) for 1 hour. The plasmid DNA band in the gel was visualized and photographed using UV-Gel Documentation system (UltraLum). Size of plasmid DNA bands was ascertained by co-running a molecular weight standard (100 bp DNA ladder plus, Fermentas Life Sciences) along with the samples in the gel.

Recombinant clones were further confirmed through separate PCR reactions using specific primer LepA and universal primer M13 (annealing temperature 52°C) in standardized reaction mixture. The amplified plasmid DNA products were separated by electrophoresis along with a MW marker (100 bp ladder plus, MBI Fermentas) using 0.75% agarose gel in TAE. The plasmids from recombinant clones were restriction with EcoR1 and Pst1 to further confirm the size of DNA fragment cloned. The reaction was performed in 20 ml, containing plasmid DNA (15ml), enzyme EcoR1 (0.5ml), Pst1 (0.5ml), 10X buffer O⁺ (2ml) and Sterile Milli-Q H₂O (2ml). Restriction product was resolved in 0.7 % Agarose gel in TAE along with a MW marker (100 bp ladder plus, MBI Fermentas).

Nucleotide sequencing of cloned DNA

Purification of sequence grade plasmid DNA: The sequencing grade plasmid DNA was isolated from the respective recombinant clone using 'Gene Elute™ Miniprep Plasmid Kit' of 'Sigma' as per manufacturer's protocol. The insert DNA cloned in these plasmids was custom sequenced through sequencing service of 'M/S Xcelris, Ahmedabad, India'.

Analysis of sequence data for taxonomic identification: For taxonomic identification, the nucleotide sequence was blasted in 'BLASTn' function of 'National Center for Biotechnology Information' (available at www.ncbi.nlm.nih.gov/Blast) and the identified based upon the derived Taxonomy report generated on the basis of maximum sequence homology/ query coverage in database from other species.

Results

Molecular characterization based on mitochondrial cytochrome oxidase I sequences

Multiple alignment among cytochrome oxidase I sequences of *H. armigera* populations (Table 3) collected from different locations identified base/nucleotide mutations at 4 different positions and all of these mutations were of substitution (Fig. 1). Out of four mutations, two involved substitution of T C (274, 313), one involved A → G (496) and one involved T → A (656).

Phylogenetic analysis of aligned sequences of mitochondrial cytochrome oxidase I revealed that Ludhiana population differed from Abohar population by the 0.2% and from Gurdaspur population by 0.7%, later two differed from each other by 0.5% (Fig. 2).

Multiple alignment among cytochrome oxidase I sequences of *H. armigera* collected from different

Table 3. Bold ID's of mt CoI sequences of various *H. armigera* populations.

Host/ Location	Identification	Specimen Page	Sequence Page	COI-5P	BIN
Tomato	<i>Helicoverpa armigera</i>	<u>Ha-L1-02</u>	<u>LEPIN063-14</u>	658	BOLD:AAA5223
Berseem Ludhiana	<i>Helicoverpa armigera</i>	<u>Ha-L1-08</u>	<u>LEPIN062-14</u>	658	BOLD:AAA5223
Berseem Abohar	<i>Helicoverpa armigera</i>	<u>Ha-G1-03</u>	<u>LEPIN061-14</u>	658	BOLD:AAA5223
Berseem Gurdaspur	<i>Helicoverpa armigera</i>	<u>Ha-Ab-07</u>	<u>LEPIN060-14</u>	658	BOLD:AAA5223
Sunflower	<i>Helicoverpa armigera</i>	<u>Ha-L1-09</u>	<u>LEPIN066-14</u>	658	BOLD:AAA5223
Cotton	<i>Helicoverpa armigera</i>	<u>Ha-L1-06</u>	<u>LEPIN065-14</u>	658	BOLD:AAA5223
Pigeon pea	<i>Helicoverpa armigera</i>	<u>Ha-L1-04</u>	<u>LEPIN064-14</u>	658	BOLD:AAA5223

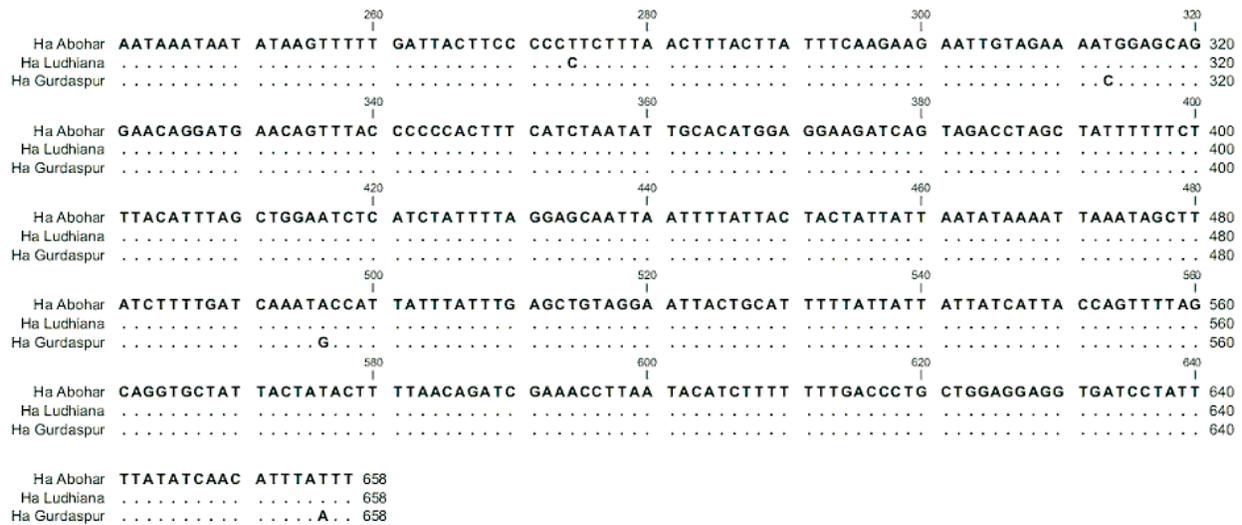


Fig. 1. Multiple alignments of mt CoxI sequences of *H. armigera* populations collected from different locations



Fig. 2. Phylogenetic tree for geographical populations of *H. armigera* infesting Berseem based on Cox I sequences

hosts identified base/ nucleotide mutations at 6 different positions and all of these mutations were of substitution (Fig. 3). Out of four mutations, three involved substitution of T C (274, 313, 350), one involved A → G (494) and one involved G → A (414,496).

Among populations infesting various host plants, based on cox I sequences cotton population differed

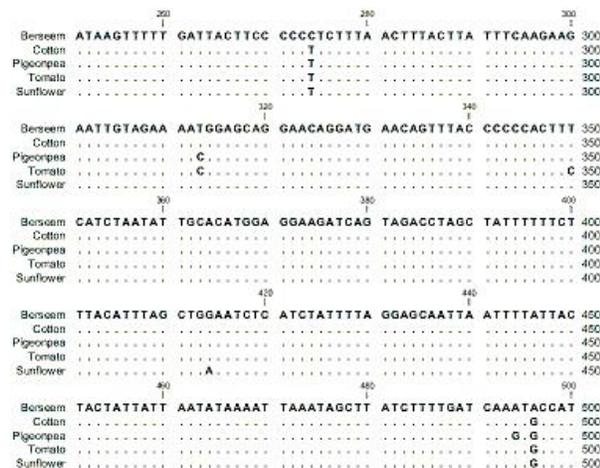


Fig. 3. Multiple alignments of mtCoxI sequences of *H. armigera* populations infesting different hosts.

by 0.2% in case of Sunflower, 0.3% in case of berseem population and 0.4% of Tomato and Pigeon pea (Fig. 4).

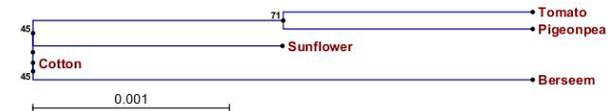


Fig. 4. Phylogenetic tree for populations of *H. armigera* infesting different hosts based on Cox I sequences

Discussion

Although there is a positive correlation between geographical and genetic distance, isolation by distance is not always expected in species with high mobility (Arguedes and Parker, 2000). Present studies found little genetic variation based on alignment of DNA sequences based on mitochondrial cytochrome oxidase I region, supporting the idea that migration was occurring in *H. armigera*. Based on cox I region, tomato and pigeon pea populations are more related and cotton is more related to sunflower and Egyptian clover populations. This may be explained as population occurring on pigeon pea might be carried over from tomato and that of cotton from sunflower and vice-versa.

The present results are in concordance with results of Australian workers who reported little variation in *H. armigera* based on allozymes (Nibouche *et al.*, 1998) and isozymes (Daly and Gregg, 1985). No obvious population substructure of Indian *H. armigera* based on EPIC-PCR markers

was indicated (Behere *et al.*, 2007). Turkish and Israeli populations of *H. armigera* also revealed low level of genetic distance (Zhou *et al.*, 2000). Substantial population substructure based on microsatellites was found in Australian populations of *H. armigera* (Scott *et al.*, 2005). In India also, many workers suggested population substructure of *H. armigera* based on RAPD markers (Yenagi *et al.*, 2012). Intraspecific populations from India form a major clade based on amino acid sequences (Asokan *et al.*, 2012). Variation in host plant properties is expected to produce changes in the life history of *H. armigera* as it has been demonstrated for many herbivorous insects (Slansky, 1993). *H. rama* was distinguished as a distinct species from within the commonly accepted *H. armigera* and collections from different host plants showed consistent differences in morphological features that merited specific separation (Bhattacharjee and Gupta, 1972).

Significant differences were revealed among *H. armigera* populations from different hosts and geographic populations with respect to fecundity and hatchability but all the populations were freely crossable with each other. Among different crosses infesting different hosts, minimum fecundity was recorded in cotton×sunflower (172.20 eggs per female) which was on par with its reciprocal cross (188.90 eggs per female) and sunflower×pigeon pea (195.30 eggs per female). Cross sunflower×pigeon pea also recorded minimum per cent egg hatch (64.11%) among all the crosses. Maximum fecundity and per cent egg hatch was recorded in cotton×pigeon pea cross (384.90 eggs per female and 79.86%, respectively). Among the crosses of *H. armigera* populations collected from different agroclimatic zones, maximum fecundity and per cent egg hatch was recorded in Central plain zone×Sub mountain zone (272.20 eggs per female and 80.12%, respectively) and minimum in case of Sub mountain zone×Western zone cross (64.25 eggs per female and 65.88%, respectively) (Gill and Arora, 2015). These results also support the idea that large scale migration is occurring among various populations of *H. armigera* which probably resulted in larger genetic dilution and low genetic differentiation.

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