Pak. J. Agri. Sci., Vol. 59(2), 289-294 ; 2022
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
DOI: 10.21162/PAKJAS/22.8288
http://www.pakjas.com.pk

Prevalence of variation among American bollworm *(Helicoverpa armigera)* collected from different host plant in Punjab-Pakistan

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American bollworm (*Helicoverpa armigera*) is poly-phagous pest which infects major fiber, pulses and vegetable crops, e.g., cotton, soybean, chickpea, brinjal, tomato, peas, beans, okra, and chili in Pakistan. Farmers use the different dosage of insecticides to control American bollworm on various crops that might be due to the existence of genetically diverse American bollworm population. So, it is essential to explore and get an understanding of this genetic variation existing among American bollworm, feeding on different host plants. The current study was conducted to observe the genetic variation existing among seven American bollworm populations by RAPD-PCR system collected from host plant including cotton, pea, green bean, chili, green chili, tomato and okra. Overall, 210-bands were obtained from thirty RAPD primers, out of these, 34-bands were polymorphic, and 176-bands were monomorphic. PIC values ranged from 0.2149 to 0.5014, with a mean of 0.2838. Genetic similarity ranged from 92.38% (produced by cotton and peas) to 99.52% (produced by chili and green beans) and the genetic distance range from 0.48% to 7.92%. A most important result was the clustering of the population occurring on green bean, chili, green chili, tomato and okra in one cluster. In contrast, the population occurring on cotton and pea were designated separate clusters, respectively. These results showed that population occurring on cotton and pea were most dissimilar to other populations indicating that populations are closely related to each other. There might be an interspecific crossing frequency among these populations.

Keywords: American bollworm, genetic diversity, host plants, insects, molecular markers, RAPD.

INTRODUCTION

The main goal of agriculture is to increase the production and quality of crops through combating diseases, insect attacks, drought, salinity and other environmental changes (Raza *et al.* 2019a; 2020). Due to insect pest attack every year, Pakistan face heavy yield losses. Different strategies can be used to evaluate the impact of these stresses physiologically and biologically (Usman *et al.* 2020). To access the molecular diversity between diverse population and individuals, stable polymorphic markers are an excellent tool (Schierenbeck, 2017; Raza *et al.* 2019b). The evaluation of molecular diversity in insect species feeding on different host plant can play an important role to get insights in infestation mechanisms of pest populations, monitoring, and their management on field crops (Fakrudin *et al.,* 2004; Gujar *et al.,* 2007).

American bollworm is one of the significant damaging insect of field crops attacking nearly 300 plant species. This is an insect with high adaptability and induced substantial yield losses in different crops like cotton, maize, sunflower, tobacco, sorghum, beats, soybean, rapeseed, chickpea, groundnut, ornamental aromatic and therapeutic plants (Shukla and Arora 2005; Rajapakse and Walter 2007). The genetic variation between genus and populations, and differential nature of *H. armigera*, cause significant yield losses in several crop plants (Scott *et al.,* 2003; Zhou *et al.,* 2000).

Evaluation of nature, extent and range of molecular diversity is important to understand the insect population dynamics, their action in response to selection pressure and

Raza, A., M. Atif, A. Ramzan, A.B U. Farooq, A. Iqbal and R.S.A. Khan. 2022. Prevalence of variation among American bollworm (*Helicoverpa armigera*) collected from different host plant in Punjab-Pakistan. Pakistan Journal of Agricultural Sciences. 59:289-294.
[Received 14 Nov 2018; Accepted 16 Feb 2021; Published 25 Apr 2022]

management. With the availability of good polymorphic markers, there is an opportunity for the correct estimation of diversity between population and individuals (Schierenbeck, 2017; Raza *et al.* 2019b). In this area, many molecular techniques have already been applied to get knowledge about genetics and evolution at the population level. One of the simple and easy technique, PCR based Random Amplified polymorphic DNA (RAPD) marker amplification is used to detect molecular divergence in many plants and insect's species (Patil *et al.* 2006; Lopes *et al.* 2017; Raza *et al.* 2019b). However, Anbalagan *et al.* (2012) described that RAPD markers are a very satisfactory approach to study the population structure and variations because it permits the observation of polymorphism exiting in nuclear and mitochondrial genomes. RAPD markers provide the real-time discovery of polymorphism at various loci in the complete genome. Therefore, it is a very effective method for studying the genetic diversity of living organisms (Raza *et al.* 2019c). In this study, we observed the genetic divergence among American bollworm populations present on diverse host plants using data developed by RAPDs in Punjab-Pakistan.

MATERIALS AND METHODS

Insect collection: *Helicoverpa armigera* adults and larvae were collected from seven different crops including cotton, green beans, tomato, chili, okra, pea and green chili present in fields of University of Agriculture, Faisalabad and from farmer's fields of the different cities of Punjab, Pakistan (Table 1) for isolation of genomic DNA. The collected specimens were washed with double-distilled deionized water and frozen with liquid nitrogen. Samples were kept at -80ºC till DNA isolation.

Table 1. Location of *Helicoverpa armigera* samples collected from different cities of Punjab province.

Sample No.	Host plant	Sampling city	Latitude - Longitude
1	Cotton	Khanewal	30.29 - 71.90
2	Green Chili	Faisalabad	31.42 - 73.09
3	Green beans	Samundary	31.06 - 72.94
4	Tomato	Chiniot	31.72 - 72.95
5	Chili	Toba Tek Singh	30.97 - 72.44
6	Pea	Kamalia	30.72 - 72.63
7	Okra	Gojra	31.15 - 72.65

Extraction of Genomic DNA: DNA from each collected sample was extracted by using CTAB method according to Bally *et al.* (2016) with slight modification. DNA was confirmed on a 1%-agarose gel to check the DNA quality. The quantification was done by using Nanodrop spectrophotometer (Thermo Scientific, Japan). Working DNA dilution of 25 ng μL^{-1} was prepared for PCR reaction.

***PCR based RAPD analysis and electrophoresis*:** Two series (GLDeca-K and GLDeca-L) of RAPD primers were used in this study for RAPD analysis, and thirty polymorphic primers were selected to amplify the genomic DNA of American bollworm (Table 2). PCR conditions were optimized by using synthesized RAPD primers for isolated DNA from insects (*H. armigera*) of different host plants. The components for 25 µl reaction mixture were as follow: 2µl-DNA template; 0.25µl-*Taq* DNA polymerase; 2.5µl 10X Buffer (+$MgCl_2$); 1µl-dNTPs (25 mM); 1µl-Primers (15 ng); 18.25µl-d_3H_2O. PCR temperature profile used is as follow: 95ºC hot start- 5 min; denaturation-95ºC for 1 min; primer annealing-34ºC for 1 min; Extension-72ºC for 2 min; Final extension-72ºC for 10 min then hold 4ºC. 3µl loading dye (0.50% bromophenole blue, 0.50% xylene cyanol) was added in products and mixed by micro-centrifuge. PCR product was electrophoresed using 2.5% agarose gel at 100V and bands which were amplified were seen on gel documentation system (Bio-Rad).

***Scoring of bands and statistical data analysis*:** All amplified bands were scored as present (1) and absent (0) for each sample. The ambivalent band that may not be cleanly described was not scored. Scored data was arranged for cluster analysis and similarity matrix. Dendrogram, genetic similarity matrix and cluster analysis for 7 populations were generated by Nei' unweighted pair group method of arithmetic mean by using popgen32 software version 1.44 (Yeh et al., 2002). Power-marker software was used to calculate polymorphism information content (PIC; Liu and Muse 2005).

RESULTS

***Information about amplified bands*:** Thirty RAPD markers of L and K series were used to measure the genetic divergence between seven American bollworm samples collected from different crops, i.e., cotton, green beans, tomato, okra, pea, capsicum and green chili. Thirty markers produced 210 bands. Out of these, 34 bands were polymorphic and showed 16.19% polymorphism and 176 bands were monomorphic and showed 83.80% monomorphism between all the measured bands. The monomorphic bands (low degree of similarity) indicated high divergence between the insect strains. Amplified bands were varying from 3-10 with a mean of 7 bands per marker. The highest number of bands (10) was amplified by primer K-18, while the lowest number of bands (3) was developed by primer L-8 (Table 2). Therefore, to maintain the repeatability and trust-ability of RAPD markers for genetic diversity studies in American bollworm and other organisms is essential. Variations in the number of bands are due to mutation, random mating and recombination during meiosis which reallocations alleles within offspring.

***Polymorphic information content (PIC)*:** PIC values were calculated for every marker. PIC was calculated to measure the discriminatory power of DNA markers for linkage studies

Table 2. Information and data developed by RAPD primers used in this study.

S. No.	RAPD Primer	Primer Sequence 5'-3'	TNABs	PBs	MBs	PIC
1	GLDeca-L.1	5'CGCAAGACCT3'	7	0	7	0.5014
2	GLDeca-L.2	5'TCGGCGTCAA3'	6	0	6	0.2149
3	GLDeca-L.3	5'CCAGCAGCTT3'	5	0	5	0.2149
4	GLDeca-L.4	5'GACTGCACAC3'	7	0	7	0.2149
5	GLDeca-L.5	5'ACGCAGGCAC3'	7	0	7	0.3248
6	GLDeca-L.6	5'GAGGGAAGAG3'	8	4	4	0.2149
7	GLDeca-L.7	5'AGGCGGGAAC3'	7	3	4	0.2149
8	GLDeca-L.8	5'AGCAGGTGGA3'	3	0	3	0.2149
9	GLDeca-L.9	5'TGCGAGAGTC3'	8	0	8	0.2149
10	GLDeca-L.10	5'TGGGAGATGG3'	7	1	6	0.3698
11	GLDeca-L.11	5'ACGATGAGCC3'	7	2	5	0.2149
12	GLDeca-L.12	5'GGGCGGTACT3'	9	2	7	0.2149
13	GLDeca-L.13	5'ACCGCCTGCT3'	7	2	5	0.2149
14	GLDeca-L.14	5'GTGACAGGCT3'	7	2	5	0.2149
15	GLDeca-L.15	5'AAGAGAGGGG3'	7	1	6	0.2149
16	GLDeca-L.16	5'AGGTTGCAGG3'	9	1	8	0.3248
17	GLDeca-L.17	5'AGCCTGAGCC3'	7	0	7	0.3248
18	GLDeca-L.18	5'ACCACCCACC3'	5	0	5	0.2149
19	GLDeca-L.19	5'GAGTGGTGAC3'	8	1	7	0.3698
20	GLDeca-L.20	5'TGGTGGACCA3'	7	4	3	0.2149
21	GLDeca-K.11	5'AATGCCCCAG3'	7	0	7	0.3248
22	GLDeca-K.12	5'TGGCCCTCAC3'	9	0	9	0.3698
23	GLDeca-K.13	5'GGTTGTACCC3'	7	2	5	0.2149
24	GLDeca-K.14	5'CCCGCTACAC3'	7	1	6	0.2149
25	GLDeca-K.15	5'CTCCTGCCAA3'	4	0	4	0.3248
26	GLDeca-K.16	5'GAGCGTCGAA3'	7	1	6	0.3698
27	GLDeca-K.17	5'CCCAGCTGTG3'	7	1	6	0.3248
28	GLDeca-K.18	5'CCTAGTCGAG3'	10	2	8	0.3698
29	GLDeca-K.19	5'CACAGGCGCA3'	7	1	6	0.4064
30	GLDeca-K.20	5'GTGTCGCGAG3'	7	3	4	0.3698
----	**Total**	**---**	**210**	**34**	**176**	**---**
----	**Percentage/Mean***	**---**	**7***	**16.19**	**83.80**	**0.2838***

Total number amplified bands (TNABs), polymorphic bands (PBs), monomorphic bands (MBs) and polymorphism information contents (PIC)

and polymorphism. PIC values were fluctuating from 0.2149 to 0.5014, with a mean of 0.2838 (Table 2). Highest PIC value (0.5014) was presented by primer GLDeca-L.1 followed by GLDeca-K.19 having a value of 0.4064, whereas the lowest PIC values (0.2149) was produced by several primers, i.e., GLDeca-L.2 to GLDeca-L.4, GLDeca-L.6 to GLDeca-L.9, GLDeca-L.11 to GLDeca-L.15, GLDeca-L.18, GLDeca-L.20, GLDeca-K.13 and GLDeca-K.14 (Table 2). Differences in PIC values were due to variations in the band size and number of amplified bands developed by every primer. Higher PIC values indicating the reliability of RAPD-PCR marker system. These results showed the ability of RAPDs to differentiate among different populations at the genomic level.

***Nei' genetic distance and similarity matrix based on UPGMA*:** Genetic distance among 7 American bollworm populations was estimated using popgene32 software version 1.44 based on Nei' Unweighted Paired Group of Arithmetic Mean Average-UPGMA (Yeh et al., 2002). Genetic similarity ranged from 92.38% to 99.52%, and the genetic distance range from 0.48% to 7.92%. Similarity, matrix data showed that insects populations from cotton and peas are 92.38% similar, peas and green beans are 97.14% similar, green bean and tomato are 97.62% similar, tomato and chili are 97.14% similar, chili and green chili are 98.10% similar, and green chili and okra are 96.19% similar (Table 2). Highest genetic distance (7.92%) was present between population collected from cotton and pea; suggesting that these two genotypes were most dissimilar to each other followed by populations collected from cotton and tomato, cotton and okra having a value of 7.41%. Meanwhile, lowest distance (0.48%) was present between populations collected from green beans and chili, which means these two populations were closely related to each other, followed by green beans and green chili having

Table 3. Genetic similarity (above diagonal) and genetic distance (below diagonal) for American bollworm populations collected from different host plant of Punjab-Pakistan.

POP ID	Cotton	Pea	Green beans	Tomato	Chili	Green chili	Okra
Cotton	****	0.9238	0.9524	0.9286	0.9476	0.9381	0.9286
Pea	0.0792	****	0.9714	0.9476	0.9667	0.9571	0.9476
Green Beans	0.0488	0.0290	****	0.9762	0.9952	0.9857	0.9762
Tomato	0.0741	0.0538	0.0241	****	0.9714	0.9619	0.9619
Chili	0.0538	0.0339	0.0048	0.0290	****	0.9810	0.9714
Green Chili	0.0639	0.0438	0.0144	0.0388	0.0192	****	0.9619
Okra	0.0741	0.0538	0.0241	0.0388	0.0290	0.0388	****

a value of 1.44%. Genetic distance results showed that all populations are somehow closely associated with each other and comparatively less dissimilar (Table 3).

Cluster analysis: The dendrogram was created to evaluate the genetic divergence among seven *H. armigera* populations from analyzed RAPD data. Cluster analysis has the singular capacity and power to judge plant /insects genotypes /populations with the highest level of similarity using the dendrogram. The insect population occurring on green bean, chili, green chili, tomato and okra were in one cluster. The population occurring on cotton and pea were together in a separate cluster, respectively (Fig. 1). Dendrogram consists of three major clusters, 1st cluster contains *H. armigera* from cotton and all other six populations, while 2nd cluster contains *H. armigera* from pea and other five populations. The third cluster consists of four sub-clusters that showed that *H. armigera* from the green bean and *H. armigera* from chili are closely related to each other and merged in one cluster. In contrast, *H. armigera* of green chili is closely related to *H. armigera* of tomato and chili. *H. armigera* from tomato is closely related to *H. armigera* from green chili and okra, whereas *H. armigera* from okra was the only one population that was closely related to *H. armigera* from tomato (Fig. 1).

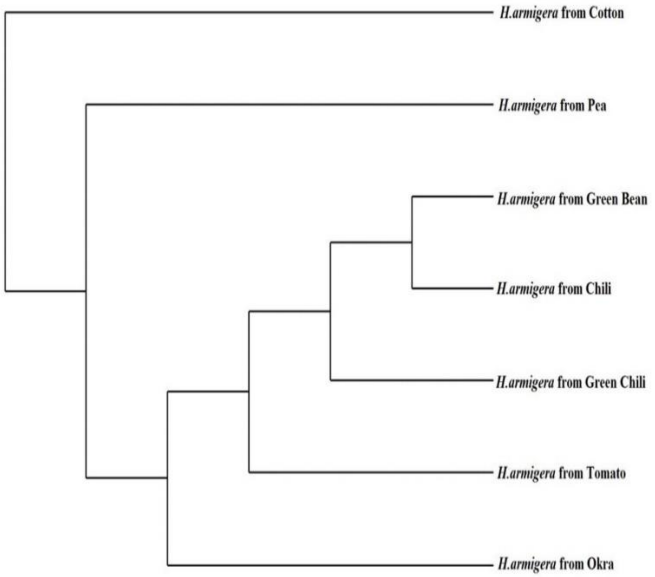

Figure 1. Dendrogram showing genetic distance of American bollworm populations feeding on different host plant.

DISCUSSION

The main focus of this research was to evaluate the genetic difference among the American bollworm feeding on different hosts plants. Genetic diversity refers to the variation exist in the genetic makeup of a species. Genetic diversity estimation among diverse populations is an important tool for genetic improvement for desirable quality, wider adaptation, pest and insect resistance that ultimately boost traditional plant breeding methods. RAPD markers can measure polymorphism, similarities, identification and grouping of genotypes. Therefore, genetic analysis of different populations of American bollworm taken from different plants is important. The study to understand the genetic variation among the American bollworm populations, feeding and present on different hosts has become essential which provide understanding about the variation in their susceptibility against insecticides. Although American bollworm is migratory and found in all crop growing continents and considered as a vital pest (Feng *et al.*, 2005; Subramanian and Mohankumar 2006). In the recent past, many scientists used RAPDs (Lei *et al.*, 1995; Zhou *et al.*, 2000; Fakrudin *et al.*, 2004; Patil *et al.*, 2006), Simple-sequence repeats (SSRs) (Tan *et al.*, 2001; Khiaban *et al.*, 2010) and Amplified fragment length polymorphism (AFLP) markers (Ming and Wang 2006) to analyze the genetic diversity and polymorphism between American bollworm populations.

In this study, we observed overall 210 bands with a mean of 7 bands per primer using 30 RAPD primers and these results are comparable with many studies like Fakrudin *et al.* (2004) measured 497 bands with a mean of 19.88 bands per primer with the help of 25 RAPD markers for 12 populations, indicating high differences between *H. armigera* populations. Whereas, Patil *et al.* (2006) observed genetic divergence between *H. armigera* present on distinct host plants in South India. They observed total of 155 bands with an average of 7.75 bands per primer using 20 RAPD markers against 10 different populations collected from different host plants.

These results are quite similar to our findings. However, our results were also supported by Lopes *et al.* (2017), who reported 117 fragments with the help of 16 RAPD markers for *Helicoverpa* populations. Observed fragments were varying from 4-12, with a mean of 7.5 fragments per marker. In another study, Zhou *et al.* (2000) measured genetic variation and population structure among 6 populations collected from different locations, and they noticed total 88 bands using 3 RAPD primers collected from 3 different sources. Khiaban *et al.* (2010) reported 46 bands with an average of 4.6 bands per marker for geographical populations by 10 SSR markers. In the current research, we observed genetic similarity ranged from 92.38% to 99.52% and the genetic distance range from 0.48% to 7.92% for seven *Helicoverpa armigera* populations collected from different regions of Punjab-Pakistan. Fakrudin *et al.* (2004) reported 0.22% to 0.42% similarity among 12 populations showing a high level of variations among studied populations. Lopes *et al.* (2017) noticed genetic distance ranged from 0.06% to 0.86% for 12 different population. Another study conducted by Patil *et al.* (2006) showed 0.25% to 0.82% similarity and 0.19% to 0.71% genetic distance among 10 different population from South India.

Huge genetic differences will be helpful for the insect population to develop and adjust to different environmental conditions. The variations in the observed number of bands, genetic similarities might be due to the relocation of populations from different sites. PIC value is used to get information about the molecular marker for linkage studies (Guo and Elston, 1999). PIC values were varying from 0.2149 to 0.5014, with a mean of 0.2838. These results showed the ability of RAPDs to differentiate among different populations at the genomic level. RAPD system may also be convenient for analysis of other insect populations.

***Conclusion*:** In conclusion, PCR-based RAPD is an advantageous technique for phylogenetic analysis, divergence analysis and molecular mapping. In the present study, we detect 210 bands using 30 RAPD markers to examine the variation among seven American bollworm populations collected from different regions of Punjab-Pakistan. The observed cluster structure required more deep studies to decide the degree of genetic diversity and behavioral variation between *H. armigera* populations existing on different host plants. Our results support that geographical separation by itself is inadequate to separate closely related populations, but natural obstacles may play a vital part in separation. In the near future, *H. armigera* populations should be collected from different environments (stressed and non-stressed plants) to know insights into the vital role of natural obstacles in yield losses caused by insects. Further, it can help in reducing the utilization of protecting chemicals like insecticides and pesticides in the natural environment.

***Acknowledgements*:** The authors are highly grateful to the University of Agriculture, Faisalabad Pakistan and farmers from different regions of Punjab-Pakistan for providing samples for this study.

***Conflict of Interest*:** The authors have declared no conflict of interest.

Author's contribution: Conceptualization, R.S.A.K. and A. R.; methodology, F.S.; validation; investigation, M. A.; resources, A.I; G.R.; writing—original draft preparation, R.S.A.K.;A.B. Farooq writing—review and editing, A. R. and supervision, R.S.A.K.; All authors have read and approved the manuscript.

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