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Genetic diversity of diamondback moth, *Plutella xylostella* populations from different host plants and across locations in North India

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Abstract
The present investigation was conducted to study the genetic diversity among different populations of *P. xylostella* populations collected from eight locations in North India [Solan, Palampur and Theog (Himachal Pradesh), Fatehgarh Sahib (Punjab), Saharnapur (Uttar Pradesh), Pantnagar (Uttarakhand), Hisar (Haryana) and Delhi] by using randomly amplified polymorphic deoxyribonucleic acid (RAPD). We used 20 primers, of which 12 primers resulted in amplified products. A total of 73.03% polymorphism was observed among the eight populations of *P. xylostella* studied. Cent percent polymorphism was observed with OPA-16 and OPAB-10 primers whereas, minimum polymorphism (40%) was observed with OPD-14 primer, which produced only 5 amplified bands. The values of similarity coefficient among all the populations ranged from 0.50 to 0.70. Maximum similarity was observed between Solan and Theog populations (70%), and between Delhi and Pantnagar populations of *P. xylostella*, which is according to the geographical distances between these locations, and the altitude of the locations from where the populations were collected. This information will be useful to develop strategies for controlling this pest through synthetic insecticides, deployment of transgenic plants for sustainable crop production.

Keywords: Diamondback moth, RAPD, polymorphism, amplification, genetic diversity

1. Introduction
The diamondback moth *Plutella xylostella* (L.) (Lepidoptera: Yponomeutidae) is one of the potential destructive pest that affects mainly cruciferous plants [1, 2, 3] pest is a prime example of the introduction of an alien species as after effect of globalization [4]. The main reason is its lack of natural enemies, enormous appetite, and high reproduction potential for its rapid spreading in short time throughout the world [5]. The larvae of Diamondback moth feed upon leaves, buds, blooms, and seed-buds of developed cole crops. The first instar larvae mine in the leaf and the subsequent instars feed on the leaf and skeletonize it ultimately affecting the plant growth and rendering it to unfit for further uses. In India, it was first reported by [5], and now it has been noticed in all members of family Brassicaceae all over India [6]. Annually, 4-5 billion US$ losses were estimated due to the management practices and crop damage [1]. Due to variations in various agro climatic factors, this insect sometimes assumes the status of major pest. It has been found by many researchers that there are considerable genetic variations among the populations of same species of insects from different geographical regions. These variations can be attributed to various evolutionary forces acting differently in different geographical regions including genetic drift, selection pressure and gene flow. The genetic variability among the populations of *P. xylostella* could be attributed to varied ecology and geography of the collection sites. Also, the insect species undergo and experience different pattern of stresses and strains caused by our farming practices [7]. RAPD markers are playing an important role in the analysis of genetic diversity of a large number of insect species. The simplicity and reproducibility of the PCR-based assays, added to their higher multiplex ratio and capacity to detect higher levels of polymorphisms which make them a lucid method to obtain intra-specific genetic variation in the insect species, where no prior sequence information is available [8].
In the face of an efficient management practices, it is necessary to know the genetic complexity and structure of the pest population, to delay the evolution of resistance to any

control method [9]. The allelic differences identified from randomly amplified polymorphic DNA (RAPD) can be used to estimate the extent of genetic differences, gene flow pattern within and between populations [10, 11]. Molecular markers provide information on the genetic diversity of diamondback moth and help to identify pesticide resistant populations in order to evolve appropriate management strategies [12]. High rates of migration between Indian populations suggest that dispersal of gene flow over considerable distances is a major factor in the development of genetic variability in the species [13]. It is important to know the genetic structure of diamondback moth from different areas before the use of large-scale efforts to control insect pests.

Therefore, the present study was undertaken to understand the genetic diversification of the different population of diamondback moth collected from different geographical areas of North India by using Random Amplified Polymorphic DNA (RAPD) studies.

2. Materials and Methods

2.1 Materials

The genomic DNA of the Diamondback moth larvae was collected from different geographical regions from February to May 2015 and was extracted by Cetyl Trimethyl Ammonium Bromide (CTAB) procedure [14]. CTAB, ethylenediaminetetraacetic acid (EDTA), sodium chloride (NaCl), chloroform, isoamyl alcohol, tris-HCl (hydrochloricacid), ethylalcohol, isopropyl alcohol 2-mercaptoethanol and RNase were used for DNA extraction. The chemicals were procured from (GeNie) Merck Private Limited, Mumbai. Taq DNA polymerase Taq DNA polymerase buffer (1x) Deoxyribonucleotide triphosphate (dNTPs), dNTP, Random primers (100 µM/ reaction) used for PCR amplification, ethidium bromide and DNA ruler were also procured from (GeNie) Merck Private Limited, Mumbai. Autoclaved distilled water was used to make solutions and washing. Muslin cloth, cotton was purchased from the local market of Solan. All chemicals were of analytical grade and used as received.

2.2 Sampling

Samples of *P. xylostella* were collected from eight different locations representing Himachal Pradesh, Punjab, Uttarakhand, Uttar Pradesh, Haryana and Delhi from different altitudes (Table 1). Each sample comprised of at least 40 larvae and 20 pupae. The larvae and pupae were collected manually from the cauliflower and cabbage fields of farmers from each location and were immediately placed in plastic jar (20 cm × 15 cm), whose, top was covered with muslin cloth with leaves of cauliflower inside the container as food to the developing larvae. These were further reared under laboratory conditions as per the method of rearing of test insect described in next section.

Table 1: Locality of collection of *Plutella xylostella*

Location	State	Altitude (mabsl)
Nauni (Solan)	Himachal Pradesh	1,275
Palampur (Kangra)	Himachal Pradesh	1,472
Theog (Shimla)	Himachal Pradesh	1,965
Fatehgarh Sahib	Punjab	260
Saharnpur	Uttar Pradesh	284
Pantnagar	Uttarakhand	243
Delhi	Delhi	216
Hisar	Haryana	215

2.3 Rearing of test insect

Collected samples were reared under laboratory conditions in wooden rearing cages of the size of 36 × 34 × 24 cm with glass pane on three sides. Fresh leaves of cauliflower with their petiole dipped in glass vials (7 cm × 1.5 cm) were kept inside the cages. Thus, the larvae collected from field were shifted to these leaves and were allowed to develop up to adults. The emerged adult were fed with 10% sugar syrup in cotton swab and provided with fresh cauliflower leaves for egg laying. The culture of the test insect collected from each location was maintained under laboratory conditions at room temperature varying from 25±1 ºC throughout the period of study.

2.4 Insect material

Three larvae of fourth instar from each location sample were picked up from the laboratory reared population of the diamondback moth for isolation of genomic DNA. These larvae were killed with chloroform and then dissected to completely remove the gut contents so that there was no contamination of plant DNA.

2.5 DNA isolation of *P. xylostella*

Only the skin and legs left after the dissection were used to extract the genomic DNA by CTAB method [14] with some modifications. Skin and legs nwere grinded in a mortar and pestle containing 700 µl of pre warmed DNA extraction buffer. After that the mixture was transferred into autoclaved centrifuge tube and incubated at 65°C for 2 h in heating water bath. During incubation samples were gently mixed at the interval of 10 min. After incubation tubes were kept at room temperature for about 10-15 min. for cooling. Equal volume of chloroform: isoamylalcohol (24:1 v/v) was added and mixed by inversions for about 10 min. to emulsify or denature the protein and facilitate the phase separation. The mixture was then centrifuged at 13,000 rpm for 10 min. at 4 °C. The upper aqueous phase was separated gently with the help of micropipettes without disturbing the interphase and transferred to another autoclaved centrifuge tube. Thereafter, 2/3rd volume of pre-chilled isopropanol was added to the obtained supernatant, mixed by tapping and kept in ice overnight for the precipitation of nucleic acids. The solution was then centrifuged at 13,000 rpm for 10 min. at 4 °C and precipitated DNA pellet was formed and supernatant was discarded. The DNA pellet so obtained was washed thrice with 70% ethanol to dissolve the soluble impurities and spun at 13,000 rpm for 15 min. at 4 °C. The DNA pellet was dissolved in 100 µl TE (Tris EDTA, 100 mM) and stored in deep freezer.

2.6 Purification of the genomic DNA

Extracted DNA was purified (with no RNA contaminants) by adding of 10 µl / 100 µl of RNase. The extracted genomic DNA was seen by gel electrophoresis (Genei Pvt. Ltd.) with a 1.2% agarose gel by employing 80 volts current to examine the quality of DNA. Depending on the concentration, the DNA samples were diluted with autoclaved distilled water to get a working solution of 20-25 ng/µl. The approximate purity of double stranded DNA was estimated by determination of the ratio of absorbance at 260 nm and 280 nm ($A_{260/280}$). This ratio is 1.8 for pure double stranded DNA. $A_{260/280}$ ratio greater than 1.8 suggest protein in sample, whereas one less than 1.8 suggests RNA contamination.

Quantification of DNA was done by using UV spectrophotometer (Perkin Elmer) at an absorbance of 260 nm

and 280 nm and calculated using Eq. 1 given below.

$$\text{Concentration of DNA} = \frac{OD260 \times 50\ ng \times 1000}{1000} \quad \text{Eq. 1}$$

As, the concentration of DNA sample was different at 260 nm, therefore each DNA sample was diluted to uniform concentration of DNA by the given formulae:

$$\frac{\text{Concentration to be needed}/\mu l \text{ X volume of DNA sample}}{\text{Concentraton of DNA sample}/\mu l \text{ at } 260 \text{ nm}} \quad \text{Eq. 2}$$

2.7 RAPD studis

RAPD reactions were carried out according to the methods [14] (Williams *et al*., 1990). Amplification of the genomic DNA of *P. xyllostella* was carried out using random decamer oligonucleotide primers. Random decamer oligonucleotide primers viz., OPA-02, OPA-03, OPA-05, OPA-14, OPA-16, OPA-18, OPAB-07, OPAB-10, OPAB-11, OPAB-19, OPAT-05, OPAT-09, OPAT-19, OPD-08, OPD-11, OPD-13, OPD-14, OPO-03, OPO-06 and OPO-07 were used for RAPD studies.

2.8 Polymerase Chain Reaction (PCR) amplification profile

The 25 µl PCR reaction mixture contained autoclaved distilled water 14.20 µl, Taq DNA polymerase buffer 2.50 µl, random primer 2 µl, dNTPs mixture 2 µl, Taq DNA polymerase 0.3 µl, genomic DNA 4 µl. The PCR was carried out in thermocycler (Eppendorf AG 22331 Hamburg mastercycler personal 5331, Made in Germany) with a total of 35 cycles. Each cycle consisted of denaturation at 94 °C for 1 min., annealing based on T_m value of primer for 1 min, extension at 72 °C for 2 min. All the PCR samples were given 5 min. time for pre PCR amplification and 10 min. post amplification at 94 °C and 72 °C respectively.

2.9 Agarose gel electrophoresis of amplified DNA

RAPD-PCR products from each location were then analyzed by gel electrophoresis. Each sample of RAPD product was mixed with 6x gel loading buffer and loaded onto an agarose (1.2 % w/v) gel which contain ethidium bromide (10 mg/ml) for electrophoresis in 1x TAE buffer (gel and tray buffer). The gel was run at 80 V for 3-4 h. A high range DNA ruler (100 bp to 10000 bp) was used as molecular standard. After electrophoresis, the gel was viewed and image was taken in Alpha-imager gel documentation system and image was saved on Compaq computer for further analysis.

2.10 Data analysis

Data analysis was carried out only for those primers which resulted in scorable patterns for the clones under study. After the amplification, the total scored bands were calculated, of which the number of monomorphic and polymorphic bands were calculated to assess the genetic fidelity of different populations of *P. xylostella*.

The individual DNA bands were scored as present or absent (1/0) in the amplification profile of all samples. Only good resolution and clear bands were scored. The scored marker data matrix was examined using the NTSYS pc-2.0 package (standard procedure). The % polymorphism was analyzed as the proportion of the polymorphic markers to the total number of markers. A dendrogram was raised after cluster analysis of the similarity coefficients by the un-weighted pair-group method analysis, UPGMA using NTSYS Pc- 2.0.

3. Results and Discussion

Of the total 20 random decamer oligonucleotide primers used, only 12 resulted in amplified DNA products. A total of 454 amplicons were observed from 12 random primers in the eight populations studied (Table 1). Solan population produced 68 amplified fragments, followed by Theog, Palampur, Saharanpur, Hisar, Fatehgarh and Delhi populations, producing 66, 58, 56, 56, 55, 50 and 45 fragments, respectively. Overall, 89 amplified bands were observed of which 22 were monomorphic, and 65 were band were polymorphic, which resulted in 73.03% polymorphism (Table 2). Minimum polymorphism (40%) was observed in OPD-14 primer, produced 5 amplified bands, and followed by OPD-13 with 50% polymorphism (Table 3). OPA-14 showed 62.50% polymorphism. There was 71.42% polymorphism in OPAB-19 and OPD-11, whereas OPA-18 and OPD-08 showed 75.00 and 77.77% polymorphism. OPA-03 and OPA-5 produced 80% polymorphism, while OPA-16 and OPAB-10 showed 100% polymorphism (Table3).

Table 2: Summary of statistics of random primers analysis for genetic diversity in *Plutella xylostella*

Parameters	Numberof amplicons
Total number of primers used	20
Total number of primers producing amplified fragments	12
Total number of bands observed	89
Total number of monomorphic bands	22
Total number of polymorphic bands	65
Per cent polymorphism	73.07
Total number of amplified fragments	454
Average number of fragments per primers	37.83

Table 3: Number of monomorphic and polymorphic fragments generated by RAPD-PCR using 12 random decamer oligonucleotide primers

S. No.	Primer Name	Total number of amplified bands	Total number of monomorphic bands	Total number of polymorphic bands	Percentage polymorphism (%)
1	OPA-02	7	2	5	71.42
2	OPA-03	10	2	8	80.00
3	OPA-05	5	1	4	80.00
4	OPA-14	8	3	5	62.50
5	OPA-16	9	0	9	100.00
6	OPA-18	8	2	6	75.00
7	OPAB-10	8	0	8	100.00
8	OPAB-19	7	2	5	71.42
9	OPD-08	9	2	7	77.77
10	OPD-11	7	2	5	71.42
11	OPD-13	6	3	3	50.00
12	OPD-14	5	3	2	40.00
Total	89	22	65	73.03	

The values of similarity coefficient ranged from 0.50 to 0.70, and maximum similarity was observed between Solan and Theog populations (70%), whereas minimum similarity was observed between Delhi and Pantnagar populations. A dendrogram based on similarity coefficients was computed by un-weighted pair-group method analysis (UPGMA) using NTSYS Pc- 2.0. Cluster analysis placed the eight populations into two main clusters, of which cluster 1 was further divided into 2 sub-clusters (Fig. 1). In sub-cluster 1, populations collected from Fatehgarh Sahib and Saharanpur depicted 66% similarity. In sub cluster 2, Solan and Theog populations showed highest similarity of 70%, whereas the population collected from Palampur showed more than 65% similarity with the other 2 populations placed in sub cluster 2.

In cluster 2, the populations from Delhi and Hisar showed 66% similarity, but >55% similarity with the populations grouped in cluster 1. The Pantnagar population showed 50% with the other populations, which was most distant to the other populations studied.

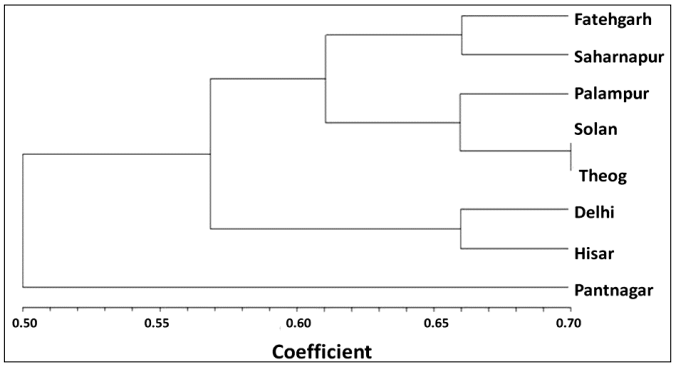

Fig 1: Dendrogram for different populations of *Plutella xylostella*

3.1 RAPD profiling for eight different populations of *Plutella xylostella*

Presence/absence of topological barriers due to environmental/weather factors and temporal barriers due to cropping patterns play an important role in genetic variability in insect populations. Diamondback moth being a migratory pest will tend to have high genetic variability within and between populations across host plants and geographical locations. Higher the genetic variability, faster is the rate of species evolution and adaption to various environmental conditions and host plants, resulting in rapid development of insect resistance to insecticides [15], and breakdown of resistance to insects in plants derived through conventional breeding and genetic transformation. In the present studies, genetic differences and high polymorphism among different populations of *P. xylostella* were observed with 12 random primers. The *P. xylostella* populations collected from different agro-climatic zones with differences in elevation and the cropping pattern might have resulted in differences their genetic makeup, as shown in Fig. 2. The maximum genetic variation was observed with OPA-8, OPA-13 and OPA-14 primers (Fig. 2 A-C). These results are in agreement to [16] who reported genetic diversity of diamondback moth populations collected from different regions of Southern India (Bangalore, Hassan, Belgaum and Shimoga) and Northern India (Delhi and Ludhiana) using RAPD markers. Five Operon (Op) primers viz., OpC 6, OpC 9, OpC 10, OpC 4 and OpB 20 produced a total of 183 amplicons. Delhi and Belgaum, Punjab and Hassan populations with 24% dissimilarities were closest, while populations with 43% genetic differences were the most diverse [15]. Studied genetic diversity in acephate, spinosad and Cry2Ab resistant *P. xylostella*collected from three states of India by RAPD markers. UPGMA analysis clustered the acephate, spinosad and Cry2Ab treated *P. xylostella*populations into two groups with overall similarity level of 33, 27 and 34%, espectively. 117 RAPD primers were used to distinguish between resistant and susceptible strains of diamondback moth, and observed that 75 primers produced one or more bands that were present in either of the strains [17]. RAPD markers are more useful in discriminating insect populations, and also for generating linkage maps to locate disease resistance genes [18]. Similar results reported [19], wherein very high variability was observed between populations of diamondback moth with 100 % polymorphism. 89.11% polymorphismalso reported [20] among the eight populations of *P. xylostella*collected from different geographical areas of China by using 15 ISSR markers.

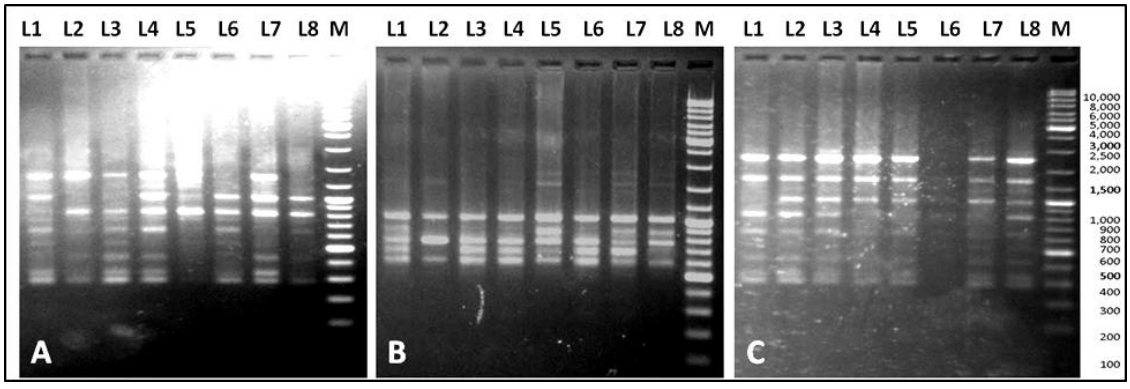

Fig 2: RAPD profile of A) OPD 8; B) OPD 13; C) OPD 14 for eight different populations of *Plutella xylostella*

4. Conclusion

Based on RAPD markers, there was considerable genetic variation in *P. xylostella* populations from different geographical locations. The populations of *P. xylostella* collected from hilly regions of Himachal Pradesh (Solan, Theog and Palampur) were genetically different from the populations collected from the Indo-Gangetic plains (Hisar - Haryana, and Fatehgarh - Punjab, Pantnagar - Uttrakhand and Delhi). This information will be useful to the designing effective strategies to control this pest through synthetic insecticides, and deployment of genetically engineered plants with resistance to this pest for sustainable crop production.

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