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PE Pakhare
Department of Plant Biotechnology, Vilasrao Deshmukh College of Agriculture Biotechnology, Latur, Maharashtra, India

Bharose AA
Department of Plant Biotechnology, Vilasrao Deshmukh College of Agriculture Biotechnology, Latur, Maharashtra, India

DG More
Department of Plant Biotechnology, Vilasrao Deshmukh College of Agriculture Biotechnology, Latur, Maharashtra, India

Thanu J Prabha
Department of Plant Biotechnology, Vilasrao Deshmukh College of Agriculture Biotechnology, Latur, Maharashtra, India

Corresponding Author:
PE Pakhare
Department of Plant Biotechnology, Vilasrao Deshmukh College of Agriculture Biotechnology, Latur, Maharashtra, India

Study of genetic variation on basis of feeding behavior of Bollwarm (*Helicoverpa armigera*) attribute molecular marker

PE Pakhare, Bharose AA, DG More and Thanu J Prabha

Abstract
Helicoverpa armigera is major important polyphagous insect pests in agriculture which attacking at least 60 cultivated and 67 wild host plants from different families and it has a worldwide distribution. The experiments were conducted to study the biology and genetic diversity of *H. armigera* (Hubner) on different host plants vis., sunflower, pigeon pea, chickpea, cotton, brinjal. The growth index and larval duration of *H. armigera* was found to be 2.96, 4.08, 2.39, 3.86 and 2.96 and 19.48, 15.99, 22.20, 17.57, 15.16 days on Sunflower, chickpea, cotton, pigeon pea, brinjal, respectively. Set of 10 SSR primers used in this study, which generated total 22 amplicons among which 21 amplicons were polymorphic, with an average of 1.2 amplicons per primer. The PIC Value ranged from 0.32 to 0.81. With highest PIC recorded in HaSSR8. The similarity coefficient between the genotypes ranged from 0.45 to 0.91. Cluster analysis in which maximum similarity occur in sunflower and pigeon pea. Whereas cotton and brinjal showed minimum similarity with each other. So, it is help to prepare the best integrated pest management strategy of *H. armigera* and understand the veriability in their susceptibility to different insecticides, including *Bacillus thuringiensis*.

Keywords: *Helicoverpa armigera*, biology, host plants, SSR primer

Introduction
Helicoverpa armigera (Hubner) is one of the most important agricultural pests throughout the world which classified as follows Kingdom: *Animalia*, Phylum: *Arthropoda*, Class: *Insecta*, Order: *Lepidoptera*, Familty: *Noctuidae*. (Anonymous 1990) [2, 3]**.** *H. armigera* is commonly known as old world bollworm which considered as omnivorous, with the larvae attacking at least 60 cultivated and 67 wild host plants from different families including *Asteraceae*, *Fabaceae*, *Malvaceae*, *Poaceae* and *Solanaceae* (Fitt, 1989, Pogue, 2004) [8] among these cotton, tomato, maize, chickpea, pigeon pea, sorghum, sunflower, soybean and groundnut (Fitt, 1989) [8] crops having agricultural importance. The larvae feed inside the host plant (fruit) therefore control measures of *Helicoverpa* spp. are difficult and also difficult to kill with insecticides because they have gained resistance to variety of insecticides (Kranthi, *et al*., 2001) [12].
Feeding behavior of *H. armigera* on different host is effect on growth and development of *H. armigera*. After emergence, females of the cotton bollworm start laying eggs within 2-6 days. They can lay between 300 and 400 eggs, which hatch in two days after oviposition. (Anonymous 1990) [2, 3]. Young larvae (third and fourth in stars) can cause up to 67% loss to cotton yields (Ting, 1986) [21]. Therefore, to prepare the best integrated pest management strategy of *H. armigera*, it is necessary to know how insects grow and develop (life history, behavior, feeding habits and their susceptibility and resistance to chemical and biological pesticides).
Molecular markers have been used to evaluate genetic similarity and estimate gene flow among insect populations. (Figueroa *et al*., 2002, Martinelli *et al*., 2006). SSR markers are better in measuring the genetic structure in *H. armigera* because of their characteristics such as coverage of multiple loci, co- dominance and high polymorphisum (Scott, *et al*., 2003) [17] than the RAPD markers. Non-availability of the DNA sequence information hampered use of SSR markers for *H. armigera*. But, many SSR markers specific for *H. armigera* have been identified (Tan *et al*., 2001; Ji *et al*., 2003) [11, 20].
Understanding the genetic variation among the *H. armigera* populations occurring on different host plants has become important to understand the veriability in their susceptibility to

different insecticides, including *Bacillus thuringiensis*. Control and management of *H. armigera* is very difficult because its having high mobility, high fecundity, high survival rate, short life span and ability to develop resistance against pesticides (Drake, 1991) [7]. Considering the above point, the present investigation were carried out to the feeding behavior of *Helicoverpa armigera*, DNA extraction from fourth to fifth instar larvae from different field crop and amplification of DNA in PCR for SSR primer.

Materials and Methods

Materials

Insects

The first and fifth instar larvae of *Helicoverpa armigera* collected from different field crops at Oilseed Research Station, Latur (M.S.) were used as experimental material.

Leaves of different host plants

Leaves of different host plants were collected from different field crops at Oilseed Research Station, Latur (M.S.) were used as experimental material.

Methodology

Biology of *Helicoverpa armigera* (Hubner) on different host plants

The studies on biology of *H. armigera* (Hubner) was carried out on five different host plants *viz*., pigeon pea, sunflower, cotton, chickpea and brinjal in a completely randomized design replicated three times respectively at the College of Agricultural Biotechology, Latur during 2019- 2020.

The initial culture of *H. armigera* (Hubner) was developed by collecting large number of larvae from the field of pigeon pea, sunflower, cotton and chickpea, brinjal. The collected larvae were reared individually in the plastic containers by feeding them plant parts of host plants every day. After emergence from the pupae, more number of female were observed than males. The freshly emerged adults were released in the oviposition cage of 50 × 30 sq.cm size covered with black muslin cloth. Ninety freshly laid eggs of *H. armigera* were obtained from the oviposition cage in order to study the biology on each of five different host plants. The newly hatched larvae were reared individually in a clean plastic container on plant parts of five different host plants. The developing green pods of chickpea, part of capitulum of sunflower, boll of cotton, fruit of brinjal and green pods of pigeon pea were used as food substrate of *H. armigera*.

The observations on larval duration, per cent larvae pupated, prepupal and pupal durations, and percent adult emergence were recorded on respective plant parts of five different host plants. The growth index was calculated by using howes (1953) formula.

$$\text{Growth index} = \frac{\text{Percent larvae pupated}}{\text{Mean larval duration}}$$

DNA isolation form *Helicovarpa armigera* larvae

The larvaes were washed thoroughly in double distilled water and the genomic DNA was prepared from larvae using a modified CTAB method (Saghai Maroof *et al*. 1984) [16]. Briefly, the cleaned larvaes were ground with 1.0 ml of cetyl trimethyl ammonium bromide buffer (CTAB) 2%, 100 mM Tris-HCI (pH 8.0), 1.4 M sodium chloride, 20 mM EDTA, 0.1% of 2-mercaptoethanol (added just prior to use) and suspended in the same buffer. The mixture was incubated at 60–65°C in a dry bath for 1 h with gentle shaking at 10 min interval. Equal volumes of chloroform: isoamyl alcohol (24: 1, v/v) was added and centrifuged at 13,000–14,000 rpm for 15 min at room temperature. The aqueous phase was transferred to a new eppendorf tube and then 0.6 volume of isopropanol was added and incubate at –20 °C for overnight. The mixture was then centrifuged at 10,000 rpm for 10 min to pellet the DNA at room temperature. The pellet was washed twice with 70% ethanol and centrifuged at 10,000 rpm for 10 min at 4 °C to pellet the DNA. The pellet was air-dried and dissolved in 50 µl of deionized sterile distilled water. The isolated DNA was treated with 25 µl of RNAase and mixed by gentle tapping to remove RNA. The whole content was incubated at 37 °C for 1 h.

Determination of quantity and quality of isolated DNA was done by spectrophotometer and then the electrophoresis was carried out at 100 volts for 1.5 hr using 1 X TAE buffer. The extracted genomic DNA of the all *H. armigera* larvae was used as template DNA for PCR.

PCR amplification

The genomic DNA from *H. armigera* larvaes collected from six different hosts was subjected to polymerase chain reaction (PCR) using 10 different SSR primers in Table 1 (Tan *et al*. 2001; Ji *et al*. 2003) [11]. PCR was carried out in individual reaction (50µl) contained 100ng of the extracted DNA, 1X PCR assay buffer (250mM Tris-HCl, 10mM KCl, 1.5mM $MgCl_2$), 100mM dNTP's, 100ng/µl each of forward and reverse primers, 1 unit of *Taq DNA polymerase*. PCR was performed with forward and reverse primers with an initial denaturation for 5 min at 94 ^{0}C, followed by 30 cycles of 94 ^{0}C denaturation for 45s, 52 ^{0}C for annealing for 45s and extension at 72 ^{0}C for 1 min. Finally the reactions were healed at 72^{0}C for 10 min.

Table 1: List of SSR primers for molecular characterization

Sr. No.	Primer		Sequence (5' to 3')	Total Nu. of base
1	HaSSR1	F	TAGGTGATTGTGGCTCAGTTTT	22
		R	CAAACCCATCAGCAAATGCAAC	22
2	HaSSR2	R	AACACCCATTGAAGTCCCATGAA	23
		R	TTCCTATGTTCACTGCTAGTT	21
3	HaSSR3	F	ATCCTTATGCTTTTAGCCGTTTA	23
		R	CAGTGGACTGCTATAGGCTGA	21
4	HaSSR4	F	TGTTACTTGGGTTTCCTGAATA	22
		R	ACCACCGACACGTGCCGACTTC	22
5	HaSSR5	F	GATAAGTTATTTCGGTTTAGTATT	24
		R	AAGTACCTAATCCGTTTTTATTC	23
6	HaSSR6	F	CATAGGAAGTGGTGAAGGGT	20
		R	CACATTCGTCTTTCATCGAC	20
7	HaSSR7	F	ACGTCGATGAAAGACGAATGTGA	23

		R	AAGCTGGTCTGTGCTGCCAT	20
8	HaSSR8	F	GCCGTAATGCCCTCAATTCTT	21
		R	TTCCCTCGGAGAGCCGT	17
9	HaSSR9	F	TAGTCTGGGAATTTTGTCTGGTGT	24
		R	CGTGCCATTGAAATAGTAAGCCAT	24
10	HaSSR10	F	TAAGTATGCCCTCGACTGTCGT	22
		R	CACTTTCCAATTAGCCTCGATGCT	24

Agarose gel electrophoresis

Agarose gel (1.5%) was prepared by dissolving 1.5 g of agarose in 100 ml 1X TAE buffer and EtBr (10 mg/ml) was added. Further 5 µl of DNA was mixed with 1µl of 6X gel loading dye and loaded on 1.5% agarose gel. The genomic DNA was resolved on 1.5% agarose gel through electrophoresis.

Resolution of amplified product

The amplified products were resolved on 2.5% agarose gel for SSR markers and 1.5% for RAPD marker at 5V/cm for 1 to 1.5hr. After electrophoresis, the gel was taken out for observation of banding pattern and photographed on a Gel Documentation System (Alpha-Innotech, USA).

Data scoring and analysis

Data analysis was performed using NTSYS-pc (Numerical Taxonomy System, Version 2.02i). The SIMQUAL program was used to calculate the Jaccard's coefficient. Dendrogram was constructed using Unweighted Paired Group Method for Arithmetic Mean (UPGMA) based on Jaccard's similarity coefficient.

Results and Discussion

Biology of *H. armigera* (Hubner) on different host plant

Egg: The incubation period of *H. armigera* was varied when reared on different host plants. However, incubation period of *H. armigera* was maximum on Chickpea (4.00 days) followed by pigeon pea (3.90 days), cotton (3.80 days), brinjal (3.36 days) and sunflower (3.00 days). The incubation period of *H. armigera* on first two and latter two host plants were various with each other. Significantly highest egg hatching to the extent of 90.00 percent was observed on pigeon pea and sunflower followed by cotton (87.00 percent), chickpea (85.66) and brinjal (80.00 percent).

According to following table, the incubation period of *H. armigera* was extended when reared on Chickpea. Yadav *et al*., (2015) [24] reported the incubation period of *H. armigera* was 3.94, 3.70, 4.50, 3.67, 3.92 days on pigeon pea, chickpea, sorghum, tomato, cotton. Akashe *et al*., (1997) [1] Reported the incubation period of *H. armigera* from 2 to 4 days on sunflower, 3 days each on Lucerne and sunflower (Patel and Koshiya, 1998a and 1998b) [14, 15]. The results in respect of incubation period of *H. armigera* reared on different host plants are in conformity with the results reported by above referred research workers.

Table 2: The mean incubation period, percent egg hatch, larval duration, percent pupation and growth index of *H. armigera* on different host plant

Name of the host plant	Mean incubation period (days)	Percent egg hatch	Mean larval duration (days)	Percent larvae pupated	Growth index (%)
Sunflower	3.00	88.00	19.48	57.46	2.96
Chickpea	4.00	85.66	15.99	65.35	4.08
Cotton	3.80	87.00	22.20	53.32	2.39
Pigeon pea	3.90	89.66	17.57	70.60	3.86
Brinjal	3.36	80.00	15.16	44.55	2.96
S.E ±	0.05	0.82	0.32	0.39	0.02
C.D at 5%	0.16	2.50	0.97	1.20	0.06
C.V (%)	2.57	1.66	3.04	1.17	1.15

Larva

According to Table 1, that significantly the shortest mean larval duration of *H. armigera* to the extent of 15.16 days was observed on brinjal and it was at per with that on 15.99 days on chickpea. It was followed by 17.57 pigeon pea, 19.48 days sunflower and 22.20 days on cotton. This indicated that among the host plants studied, longest larval period of *H. armigera* was recorded on cotton while shorted was on brinjal.

The larval duration of *H. armigera* was reported 18 to 21 days on sunflower (Coaker, 1998) [6], 13 to 16 days on chickpea (Menolanche *et al*., 1959), 20 to 22 days in cotton (HSU *et al*., 1960) [10]. The longest period (25.3 days) on cotton observed by Gaikwad *et al*., (1977) [9]. The larval development of *H. armigera* was completed in 18.98, 18.24, 19.43, 16.32, 18.32, 19.45, 16.4, 16.67 and 20.12 days on carnation, pigeon pea, chickpea, sorghum, tomato, cotton, cowpea, bathua and capsule of castor observed by Yadav *et al*., (2015) [24].

The significantly lowest pupation of *H. armigera* was recorded on brinjal (44.55 percent) followed by cotton (53.32 percent). The growth index values varied from 2.39 to 4.08. The significantly highest growth index was observed in the case of larvae fed on chickpea (4.08) over pigeon pea (3.86).

The data in Table 2 indicated the instars- wise larval duration of *H. armigera* when reared on different host plants. The duration of I, II, III, IV, V, VI larval instars ranged from 1.53 to 3.22, 2.00 to 3.60, 2.45 to 3.50, 2.98 to 4.05, 3.18 to 4.10 and 2.82 to 3.73 days, respectively on different host plants under investigation. The mean larval instar duration was lowest on brinjal (2.52) and maximum on cotton, followed by chickpea (2.66), pigeon pea (2.92), sunflower (3.24). Jha *et al*., (2012) [23] reported that the average first, second, third, fourth, fifth and sixth larval instar duration was 2.08, 3.77, 2.53, 4.22,5.32 and 4.71 days, respectively on hybrid sweet corn.

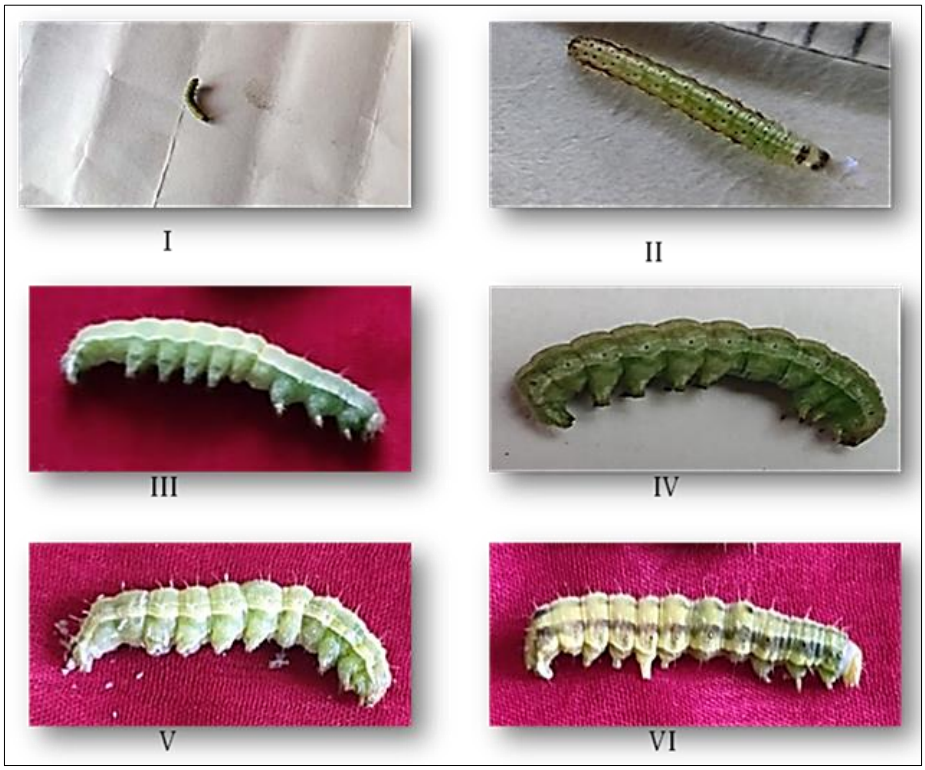

Fig 1: Larval instars of *H. armigera* (Hubner)

Table 3: The mean larval instars duration of *H. armigera* on different host plants

Name of the host plants	Duration (days)						Total duration (days)	Mean duration (days)
	Larval instars							
	I	II	III	IV	V	VI		
Sunflower	3.17	2.92	3.33	3.09	3.45	3.52	19.48	3.24
Chickpea	1.63	2.00	2.45	3.15	3.50	3.26	15.99	2.66
Cotton	3.22	3.60	3.50	4.05	4.10	3.73	22.20	3.70
Pigeon pea	2.38	2.78	3.14	2.98	3.23	3.06	17.57	2.92
Brinjal	1.53	2.11	2.45	3.07	3.18	2.82	15.16	2.52
S.E±	0.09	0.07	0.06	0.10	0.11	0.07	-	-
C.D at 5%	0.28	0.23	0.18	0.30	0.36	0.21	-	-
C.V (%)	6.80	4.96	3.58	5.31	5.88	3.78	-	-

Pupa

The pre pupal and pupal duration, percent adult emergence and total developmental period of *H. armigera* on different host plants are presented in Table 3.

Table 4: The mean pre pupal and pupal duration, percent adult emergence and total development period of *H. armigera* on different host plants

Name of the host plants	Pre-pupal duration (days)	Pupal duration (days)	Per cent adult emergence	Total development period (days)
Sunflower	2.26	15.11	69.60	39.87
Chickpea	2.07	9.09	72.21	31.16
Cotton	2.52	11.31	65.60	39.85
Pigeon pea	2.11	9.55	71.46	33.14
Brinjal	2.02	11.78	60.09	31.73
S.E ±	0.04	0.13	0.48	0.47
C.D at 5%	0.14	0.41	1.48	1.44
C.V (%)	3.76	2.09	1.24	2.34

The significantly shortest mean pre pupal duration of *H. armigera* to the extent of 2.02 days was recorded on brinjal while shortest mean pupal duration to the extent of 9.09 days was recorded on chickpea. While the pre pupal duration of *H. armigera* were highest on cotton and pupal duration of *H. armigera* were highest on sunflower (15.11) followed by brinjal (11.78), cotton (11.31), pigeon pea (9.55) and chickpea (9.09).

Patel and Talati (1987) [13], Tripathi and Singh (1989b), Anonymous (1990) [2, 3], Shrivastava and Shrivastava (1990), Singh *et al*., (1992) [18], Bajpal and Sehgal (1993) [4], Choudhary *et al*., (1993) [5] and Venkataiah *et al*., (1994) [23] observed that the pupal duration of *H. armigera* was 9 to 17 days on sunflower, 7.8 to 21.2 days and 11.3 to 24.45 days on pigeon pea and sunflower, 9.55 days on cotton cultivars, 9.0 and 7.1 days on gram leaves and pods, 14.0, 12.3, 10.3 and 14.3 days on chickpea, sweet pea, pigeon pea, and lentils and 9.3 and 9.6 day on chickpea and blackgram, respectively. The

results on the pre pupal and pupal period of *H. armigera* on different host plants in good line with above referred research workers. The highest adult emergence was observed in chickpea (72.21 percent) followed by pigeon pea, sunflower, cotton and brinjal (Table 3). This indicates that the chickpea was suitable host for emergence of adults.

Molecular characterization by using SSR primers of *H. armigera*: A set of 10 SSR primers were used to carry out PCR amplification of 5 genomic DNAs. Following SSR primers were found to be polymorphic, monomorphic and selected for final PCR amplification. They are listed in Table 5. The dendrogram Figure 1 showing phylogenetic relationship based on UPGMA cluster analysis reveals that the genotype under present study could be divided into two subcluster *viz*., cluster A and cluster B. cluster A consist of Cotton. Cluster B is divided into B1 and B2. B1 is further grouped into B1a and B1b. Sub-subcluster B1a consist of Sunflower and Pigeon pea. Sub-subcluster B1b consist of Chickpea. Subcluster B2 is consist of Brinjal.

Table 5: List of *H. armigera*'s host plant for cluster analysis with DNA based SSR markers

Name of cluster	Name of sample		
Cluster A			Cotton
Cluster B	B1	B1 (a)	Sunflower
			Piegon pea
		B1 (b)	Chickpea
	B2		Brinjal

In the present study using SSR primers a total number of 25 amplicons were generated by 10 SSR primers Table 6. 21 amplicons were found to be polymorphic with an average polymorphism of 66.7 percent. On an average each primer produced 3.1 amplicons. The size of amplification product ranged from 300bp-100bp.

1. The SSR Primer HaSSR2 produced all monomorphic bands on 200bp. Hence showed 0% polymorphism.
2. The SSR primers $HaSSR_3$, $HaSSR_4$, $HaSSR_5$, $HaSSR_7$, $HaSSR_8$ and $HaSSR_{10}$ produced 2 amplicons each. All or one the amplicons generated were polymorphic. The fragment size ranged from 300bp-100bp.
3. The SSR Primer $HaSSR_1$, $HaSSR_6$ and $HaSSR_9$ Produced maximum number of amplicons (3). All were polymorphic in nature. The fragment size is ranged from 300bp-150bp.
4. Similarity coefficient values obtain from SSR molecular marker data analysis.

The dice similarity coefficient of *H. armigera* on different host plant represented in Fig. 1 ranged from 0.45 to 0.91 indicating that high diversity was present among those *H. armigera* on different host plant. Maximum similarity coefficient of 0.91 was observed between Sunflower and Pigeon pea. And minimum similarity coefficient was observed between Cotton and Brinjal.

The PIC value generated by SSR primer ranged from 0.32 to 0.81. Highest PIC value recorded in primer $HaSSR_8$ (0.81). Whereas minimum PIC value recorded in Primer $HaSSR_5$ (0.32).

Subramanian, S and Mohankumar, S. (2006) [19] reported among the ten SSR primer, nine SSR primers indicated high variability across the different host with polymorphism ranging from 75 to 100 percent. Result was the primer HaSSR2 produced a single monomorphic band for all DNA samples of different host plants. The coefficient values ranged from 0.348–0.741. The *H. armigera* populations occurring on tomato and bhendi were found to be closely related with a coefficient of 0.741, while the population occurring on cotton and blackgram was found to differ widely with a coefficient value of 0.348. The population on cotton was found to be distantly related to the others with lower dice coefficients.

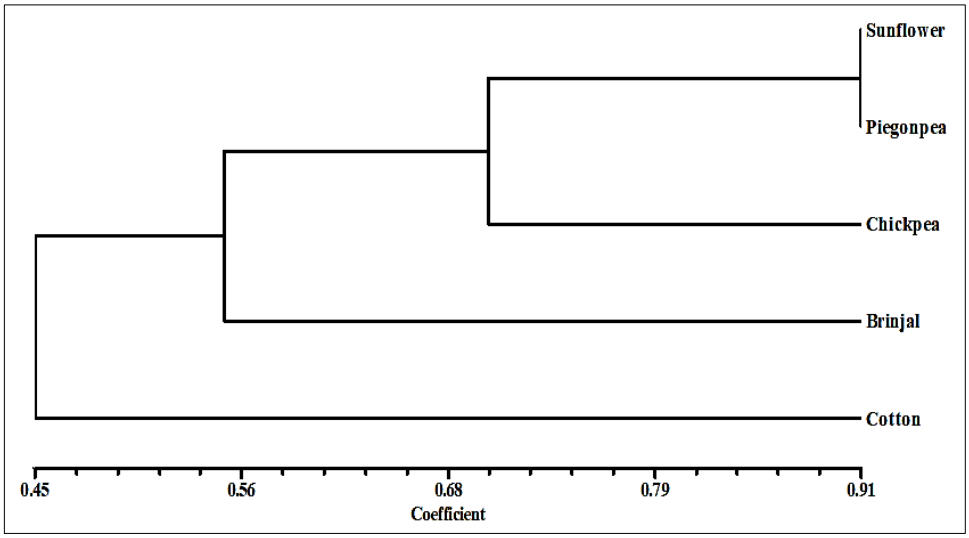

Fig 2: Dendrogram of *H. armigera* on different host plants by SSR Markers

Table 6: Polymorphic SSR primers used for analysis of *H. armigera* on different host plants

Name of primer	Annealing temp °C	No. of amplicons generated	Polymorphic amplicons	Monomorphic amplicons	Size of amplification product	Percent polymorphism	PIC value
HaSSR1	52	3	3	-	300bp-100bp	100	0.52
HaSSR2	52	1	0	1	200bp	0	0.74
HaSSR3	52.5	2	1	-	350bp-100bp	50	0.64
HaSSR4	52	2	1	-	250bp-150bp	50	0.57

HaSSR5	53	2	2	-	250bp-150bp	100	0.32
HaSSR6	52	3	3	-	300bp-200bp	100	0.46
HaSSR7	54	2	2	-	300bp- 200bp	100	0.32
HaSSR8	52	2	1	1	200bp- 250bp	50	0.81
HaSSR9	54	3	2	-	350bp-150bp	67	0.47
HaSSR10	58	2	1	-	300bp-200bp	50	0.22

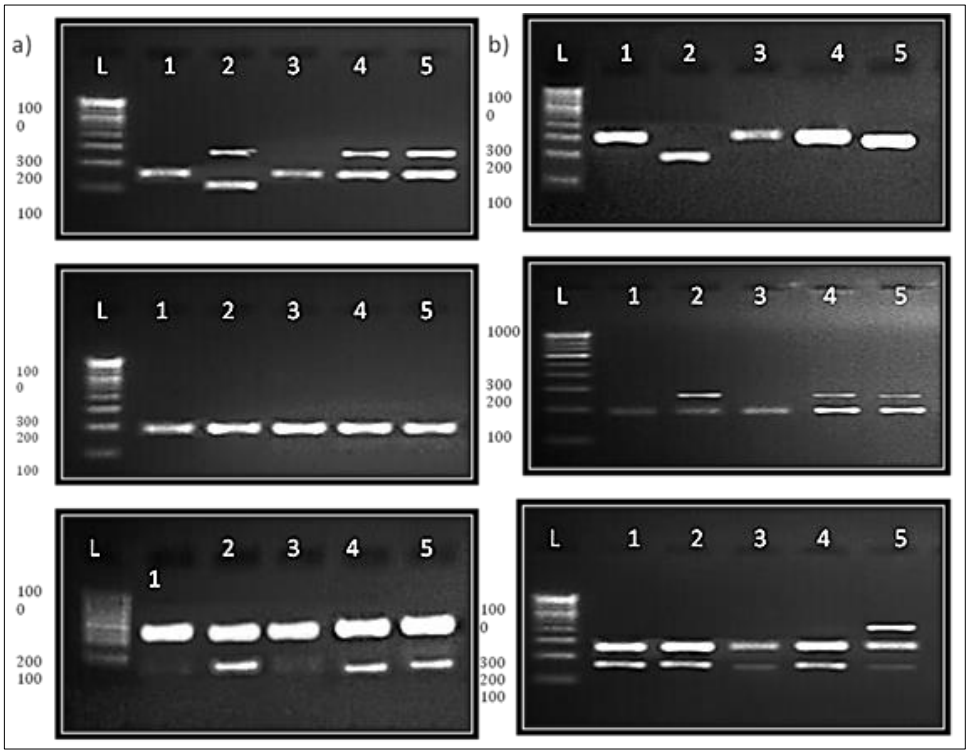

Fig 3: A SSR profile of *H. armigera* on different host plant with primer a) HaSSR1,, HaSSR 2, HaSSR3; b) HaSSR7, HaSSR8, HaSSR9, compared with 1 Kb DNA ladder. (L- Ladder, 1- Sunflower, 2- Cotton, 3- Pigeon pea, 4- Chickpea, 5- Brinjal)

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