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SHIFT OF *HELICOVERPA ARMIGERA* FROM ONE HOST TO ANOTHER AFFECTS GUT BACTERIAL DIVERSITY

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ABSTRACT: *H. armigera* is one of the constraints known to reduce yield of economically important crops, which harbor rich bacterial diversity in the gut. Hence present work deals with the microbial community study from gut *of H. armigera* when shifted from one to another hosts. By using PCR 16S rDNA was amplified and analyzed by denaturing gradient gel electrophoresis (DGGE). The resulting PCR-DGGE band number was counted, and the banding patterns were analyzed by calculating the Jaccard pairwise similarity coefficients (Cs). Shannon indices showed highest diversity when larvae fed on pigeonpea. But the shifting from pigeon pea to other host resulted in to the decrease in the diversity. On the contrary diversity was found to be increased when larvae shifted from other hosts to pigeonpea. This study concludes that shift of the larvae from one host to another result into the change in gut microbial diversity. Detail understanding of this change in diversity may help to design rational strategy for the control of pest attack.

KEYWORDS: *H. armigera*, gut bacterial diversity, PCR, DGGE

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1. INTRODUCTION

H. armigera is a major pest of food, oilseed, fodder, and horticultural crops. *H. armigera* has a worldwide distribution including Africa, Asia and some countries in Europe (Tay et al. 2013). It is commonly known as Cotton Bollworm or American Bollworm. *H. armigera* is a polyphagous and cosmopolitan insect found to infest more than 300 agricultural important crop plants and leads to massive loss (Rajapakse and Walter 2007). The polyphagy, wide host range, high mobility and high fecundity allows the pest to breed throughout the year leading to massive loss (Deepa and Srivastava 2010). Heinrich Anton de Bary in his monograph "Die Erscheinung der Symbios" introduced the term symbiosis (DeBary 1879). Most of living organisms are known to posses gut microbes for their digestion and other activities (Douglas 2011). Role of symbionts in speciation due to the rapid evolution of host immune genes in response to microbial symbionts is reported (Brucker and Bordenstein 2012). Xiang et al. (2006) has shown that larvae of *H. armigera* collected from field harbors major diversity of gut bacteria as compared to laboratory reared larvae. Furthermore, Gayatri et al. (2012) revealed that the diversity of bacteria present in the leaf phyllosphere of the host plant has significant contribution in the diversity of *H. armigera* gut bacterial population. Gayatri et al. (2012) also observed the presence of some universal bacterial phylotypes besides varied diversity in the gut of *H. armigera*, irrespective of the change in host plant and location. Therefore the evaluation of common/ permanent and diverse gut bacterial flora is important. In the present study, we used DGGE to determine the extent of bacterial diversity. We isolated gut bacteria from field collected *H. armigera* larvae using a PCR based culture independent method. For distinguishing bacterial diversity denaturing gradient gel electrophoresis (DGGE) has advantages over culture methods (Dillon and Dillon 2004). For characterizing phylloplane and rhizosphere microbial populations this method is used in plant protection research in studying gut microflora (O'Callaghan et al. 2003). The aim of the present study was to know the variation in the gut microbial diversity during shift of *H. armigera* populations from one to another host plant.

2. MATERIALS AND METHODS

2.1 Collection of larvae

The 2nd and 6th instar larvae of *H. armigera* were separately collected from cotton, pigeonpea and chickpea fields located near Aurangabad city, Maharashtra, India. For microbial analysis after shifting, one group (10 larvae in each group) of 2nd instar larvae was moved from one host to other in following manner. Two groups of larvae native to pigeonpea were transferred to cotton and chickpea and two groups of larvae native to cotton transferred to pigeonpea and chickpea. Finally two groups from chickpea are shifted to cotton and pigeonpea. These larvae with altered host allowed growing up

to the 6th instar.

2.2 Dissection and DNA extraction

For identification of gut bacterial communities, the guts of larvae feeding on its native host plants and altered host plants were assessed for microbial communities. Ten larvae of 6th instar were dissected with alcohol-sterilized dissecting tools and guts were removed carefully. The isolated guts were placed in sterile micro centrifuge tubes on the ice.   DNA was extracted with a QIAamp DNA Stool Mini Kit (QIAGEN, USA). The extracted DNA was quantified with Eppendroff Bio Spectrometer® and stored at −20 °C

2.3 PCR Amplification

The 16S rDNA of gut bacteria was amplified by polymerase chain reaction (PCR). The primers specific for conserved sequences (variable V3 region of the 16SrDNA): 343f (5′-ACTCCTACGGGAGGCAGCAG-3′) and 534r (5′-ATTACCGCGGCTGCTGG-3′) were as per protocols used by Nakatsu et al. (2000) and Xiang et al. (2006). The DNA samples obtained from the gut of 6th instars field collected larvae were used as templates for comparisons among different host population. The prevention of strand dissociation and stable PCR product during DGGE was achieved by the addition of a G+C clamp (5′-CGCCCGCCGCGCG CGGCGGGCGGGGCGGGGGCCCGGGGG-3′) to the 5′ end of the forward primer. Total reaction volume of 50µl containing DNA extract (5 µl), each primer (5 pmol), dNTP mixture (1.8 µl: 2.5 mmol/mL for each), Taq PCR reaction buffer (2.5 µl 10×) and r*Taq* DNA polymerase (1 unit) (sigma) subjected to PCR amplification. The optimized conditions for PCR thermal cycles for the universal primers were used as: initial denaturation at 95 °C (5 min), 35 cycles of 94 °C (30 sec), annealing at 55 °C (30 sec), extension at 72 °C (30 sec) and extension at 72 °C (10 min). Purification of the PCR products was performed using Quick PCR purification kit (Bangalore GeNei).

2.4 Denaturing Gradient Gel Electrophoresis (DGGE)

The PCR products were analyzed by DGGE using a Bio-Rad D-code system. It was performed using 8% (m/v) gels of acrylamide (acrylamide-bis-acrylamide, 37.5:1 m/m) with a 30% to 70% of denaturant (100% = 7 mol/L urea and 40% deionized formamide (v/v)) as per Xiang et al. (2006). The resultant PCR products were concentrated, mixed with gel loading buffer and subjected to electrophoresis at a constant temperature of 60 °C for 12 hr at 70 V. After electrophoresis, silver staining was used as per Joshi et al. (2013) to visualize banding pattern on DGGE gel. UVITEC Gel Doc system was used to capture and analyze silver stained DGGE gel image.

2.5 Statistical analysis

The statistical analysis of all the data obtained by DGGE experiments was performed using PAST (Paleontological Statistic Software ver. 1.88).

3. RESULTS AND DISCUSSION

In all shifting cases a similar DNA profile was observed with slight differences with respect to the intensity of bands. An intense single band of 250bp was observed in all the nine wells along with DNA marker as shown in figure 2.1
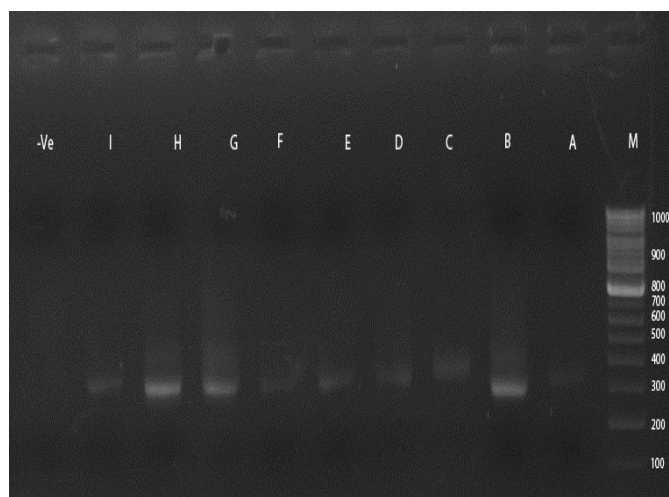

Figure 2.1 Amplification of V3 region of 16S rDNA

Lanes M- represent DNA ladder (GeneRuler[TM] 100bp DNA ladder) and lanes A-I represent samples of microbial DNA present in the mid-gut of *H. armigera* collected from   A) cotton B) chickpea C) pigeonpea D) cotton to pigeonpea E) cotton to chickpea F) pigeonpea to cotton G) pigeonpea to chickpea H) chickpea to cotton I) chickpea to pigeonpea.

3.2 DGGE technique

The guts of actively feeding 6th instar larvae of *H. armigera* collected from the cotton, chickpea and pigeonpea fields were subjected for genomic DNA extraction and analysis. The 16S rDNA universal primers were used for the profiling of bacterial communities using PCR DGGE. The genomic DNA obtained was amplified by using a nested PCR approach under conditions described in materials and methods. The separation of the amplified 250bp 16S rDNA fragments of gut bacteria of *H. armigera* was carried out using DGGE (Fig.2.2)

Figure 2.2 DGGE profile of 16S rDNA.

The separation of the 250bp 16S rDNA fragments of gut bacteria of *H. armigera* using DGGE.30-70% denaturing gradient was used on an 8% polyacrylamide gel. A) cotton B) chickpea and C) pigeonpea D) cotton to pigeonpea E) cotton to chickpea F) pigeonpea to cotton G) pigeonpea to chickpea H) chickpea to cotton I) chickpea to pigeonpea.

3.3 Analysis of the DGGE banding profiles

The variation in the bacterial community was studied using DGGE banding profiles as per Muyzer et al. (1993). The comparison of DGGE banding profiles was carried out using PHORETIX 1D gel analysis software (version 4.0, Phoretix International, Newcastle upon Tyne, UK) on the basis of presence or absence of bands at definite positions in each lane (Tourlomousis et al., 2010). DGGE profile of 16S rDNA fragments amplified by PCR showed variation in microbial population. When insect were fed on cotton, in total 5 light and intense bands were detected. When larvae were fed on chickpea 18 light and intense bands were detected. But when larvae were fed on pigeonpea 35 bands were detected on DGGE. The variation in the microbial communities was observed when insects shifted from 1) cotton to pigeonpea and chickpea, 2) pigeonpea to chickpea, cotton 3) chickpea to pigeonpea and cotton. Total 13 and 7 distinct bands were detected when larvae were shifted from cotton to pigeonpea and chickpea respectively. When larvae were shifted from pigeonpea to chickpea and cotton 18 and 7 bands were detected respectively. Whereas, when larvae were shifted from chickpea to pigeonpea and cotton respectively 14 and 10 bands were detected. Total 4 common bands were detected in all the shifts.

3.4 Statistical analysis of the DGGE

Statistical analysis of the DGGE data using similarity matrices showed significant variation in the bacterial communities between the samples. The range of 0.11 to 0.71 was observed for Jaccard coefficients for all the samples (Table 2.1). As per the pairwise comparisons, the shifts Chickpea-Cotton and Chickpea-Pigeonpea shared the maximum similarity (0.71) in bacterial composition. Cotton and pigeonpea shared the lowest similarity (0.11). The DGGE data was also subjected to Correspondence Analysis (CA). It is a graphical representation of similarity. For two-way contingency table, the DGGE profiles of midgut bacterial population from each shift were taken as sample point and DGGE bands defined as variables. The Eigen Axis 1 and Eigen Axis 2 showed the variance of 40.08% and 17.29.50% respectively. Total 57.38% of variance in bacterial composition was calculated between the shifts (Fig. 2.3).

Host	P	P - Chi	P - C	Chi	Chi - P	Chi - C	C	C- P	C - Chi
P	1								
P - Chi	0.35897	1							
P - C	0.16667	0.31579	1						
Chi	0.325	0.5	0.31579	1					
Chi - P	0.32432	0.52381	0.4	0.52381	1				
Chi - C	0.25	0.47368	0.54545	0.47368	0.71429	1			
C	0.11111	0.21053	0.33333	0.27778	0.26667	0.36364	1		
C- P	0.2973	0.40909	0.33333	0.55	0.42105	0.4375	0.38462	1	
C - Chi	0.16667	0.25	0.4	0.31579	0.3125	0.41667	0.33333	0.25	1

Table 2.1 Pairwise comparison for similarity of DGGE from the gut of *H. armiger* larvae collected on different host plants.

P: pigeonpea, P-Chi: pigeonpea to chickpea, P-C: pigeonpea to cotton, Chi: chickpea, Chi-P: chickpea to pigeonpea, Chi-C: chickpea to cotton, C: cotton, C-P: cotton to pigeonpea, C-Chi: cotton to chickpea (P – Pigeonpea, C – Cotton, Chi – Chickpea).
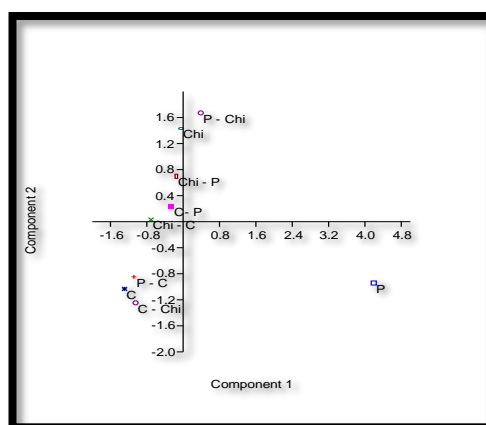

Figure 2.3 CA analysis of the gut bacterial diversity of *H. armigera* from different hosts.

The similarity between the band patterns was calculated by matching the band positions. The cluster showed an interesting nested cluster structure indicating the presence of similar or common flora with the different levels of variations in them.   The smaller cluster consisted of chickpea and pigeonpea representing the close relation in-between the two. This cluster in-turn shared the similarity with the crops pigeonpea-chickpea and another group of the crops of chickpea-pigeonpea and chickpea-cotton. This cluster indicates that the variation in the microflora of these crops due to the shift is not differing significantly or they share similar microflora. The comparison of this cluster with the basic crops showed the decrease in the branch length implying the reduction in the range of microflora but sharing the microflora present within these basic crops. The cluster again showed the relation with another cluster of pigeonpea-cotton and cotton-chickpea showing close relatedness with each other with high amount of similar microflora and sharing the similarity. The basic microflora of cotton and pigeonpea represent higher branch length as compared to all the other implying the presence of higher microflora than that of the other entire crops (Fig 2.4). The Shannon H index showed that the high population pigeonpea had the highest diversity, 3.555, followed by pigeonpea to chickpea (2.89) and chickpea (2.89), chickpea to pigeonpea (2.639), cotton to pigeonpea (2.56), chickpea to cotton (2.30), pigeonpea to chickpea (1.94), cotton to chickpea (1.94), cotton (1.60)   (Table 2.2). Similarly the Simpson index and Chao-1 index were high for Pigeonpea (1-D =0.97, Chao 1 = 630), followed by pigeonpea to chickpea and chickpea, and chickpea to pigeonpea (Table 2.2). The gut microbial diversity analysis showed that pigeonpea population is more diverse.
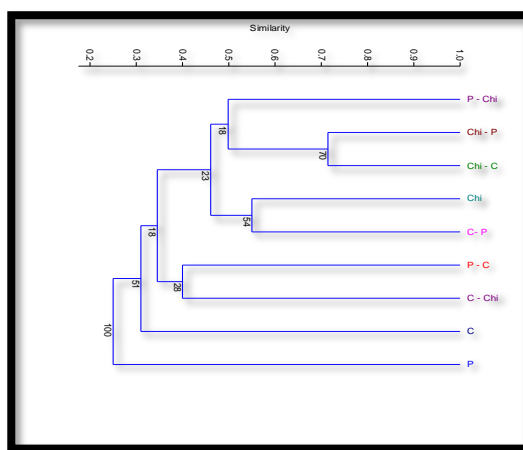

Figure 2.4 UPGMA clustering analysis of DGGE pattern of bacterial community profile.

P: pigeonpea, P-Chi: pigeonpea to chickpea, P-C: pigeonpea to cotton, Chi: chickpea, Chi-P: chickpea to pigeonpea, Chi-C: chickpea to cotton, C: cotton, C-P: cotton to pigeonpea, C-Chi: cotton to chickpea.

	P	P - Chi	P - C	Chi	Chi - P	Chi - C	C	C- P	C - Chi
Taxa_S	35	18	7	18	14	10	5	13	7
Individuals	35	18	7	18	14	10	5	13	7
Dominance_D	0.02857	0.05556	0.1429	0.05556	0.07143	0.1	0.2	0.07692	0.1429
Simpson_1-D	0.9714	0.9444	0.8571	0.9444	0.9286	0.9	0.8	0.9231	0.8571
Shannon_H	3.555	2.89	1.946	2.89	2.639	2.303	1.609	2.565	1.946
Chao-1	630	171	28	171	105	55	15	91	28

Table 2.2 Diversity indices of gut bacteria populations of *H. armigera* larvae

P: pigeonpea, P-Chi: pigeonpea to chickpea, P-C: pigeonpea to cotton, Chi: chickpea, Chi-P: chickpea to pigeonpea, Chi-C: chickpea to cotton, C: cotton, C-P: cotton to pigeonpea, C-Chi: cotton to chickpea

DISCUSSION

It is hypothesized that gut bacterial community plays important role in the crucial metabolic processes of the host insect (Dillon and Dillon 2004). Also the colonization of dominant nonpathogenic microbes restricts insect pathogens (Xiang et al. 2006). So far, symbiotic relationship between insects and bacteria is largely explored in Lepidoptera. Within the Lepidoptera, *H. armigera* is a polyphagous pest of several economically important agricultural crops (Ryan 1990). Recently the diversity of microflora from the gut of *H. armigera* has been studied. Significant variation of bacterial diversity was observed with respect to different host plants and places (Gayatri et al. 2012). In our earlier study we have reported protease producing *bacillus* sp. in gut of *H. armigera* (Shinde et al. 2012). But as per our knowledge, no reports are available on the gut bacterial diversity when larvae shifted from one host to another. Gayatri et al. (2012) has showed that the enzymes synthesized by gut bacteria have crucial role in encountering the plant defense. Therefore the successful association between microbes and their insect host is important for the adaptation of the insect to its surrounding environment (Dillon and Dillon 2004).   Thus, gut bacteria have crucial role in the resistance of *H. armigera* to insecticides and pesticides.

H. armigera is rapidly overcoming present control measures, such as chemical and microbial insecticides along with genetically modified plants (Kranthi et al. 2002; Rajgopal et al. 2009), hence targeting the gut bacteria will be an effective strategy to control the attack of *H. armigera*. However the varying diversity of gut bacteria may have repercussions on the efficacy of this strategy. Therefore, it is necessary to find out whether the shifting of *H. armigera* from one host crop to another affects the diversity of gut bacteria. Our culture independent molecular analysis showed the presence of significant diversity in gut bacterial population of the larvae collected for each shift (Table 2). Recent studies have revealed that gut bacterial community is largely environmentally acquired, and some transmitted socially (Oliver and Martinez 2014). Therefore diverse population observed in each shift

could be the one which was present on the phyllosphere of the respective host plants before and after shifts. According to some researchers, insects lack specialized structures of the digestive system (Appel 1994; Bignell and Eggleton 1995). In addition, ingested food has rapid transit through digestive system. Therefore ingested bacteria from the host plants phyllosphere flora may have minor contribution in the insect's digestion (Appel 1994; Bignell and Eggleton 1995). However, the gut bacterial flora of *H. armigera* collected for each shift showed some similarities (Table 1). Based on the DGGE banding profiles obtained, the bacterial communities between shifts were compared by Correspondence analysis (Fig. 3). The shifts sharing similar bacterial flora were aligned together by cluster analysis which revealed three major clusters for DGGE profile (Fig. 4). The presence of common and stable bacterial phylotypes in the gut of *H. armigera* larvae collected from the native host and after shifting suggests that they could be the longer and dominant residents of the insect gut. This is also in correlation with the observation that some bacterial species form common and stable colonizers in the insect gut which is independent of plant diet (Broderick et al. 2004; Behr and Kapur 2008; Tang et al. 2012). The presence of such universal phylotypes in all the shifts is in agreement with earlier reports of Xiang et al. (2006) and Gayatri et al. (2012). Besides the *H. armigera*, the dominance of Proteobacteria and Firmicutes along with Actinobacteria in the gut of several herbivorous insects is observed (Schloss et al. 2006; Pittman et al. 2008; Hernandez et al. 2014; Shao et al. 2014; Dantur et al. 2015). It has been demonstrated that stable symbiotic bacterial community increases longevity of the insect host by contributing in the digestion, detoxification and development of the insect host (Behr and Kapur 2008; Vissoto et al. 2009; Oliver and Martinez 2014). Therefore it is necessary to evaluate the physiological role of stable, long retained gut bacterial flora in the *H. armigera* for their contribution in the insect's health. So it can be targeted / applied accordingly for the control of *H. armigera* infestation.

4. CONCLUSION

In conclusion we have studied whether the shift of *H. armigera* from one host to another would affect the gut microbial population of *H. armigera*. This study shows that along with change in the diversity *H. armigera* retains some common and stable gut bacterial flora after shifting from one host to another. This suggests that retained flora may have some contribution in some physiological processes of the host. Detailed understanding of this contribution may help us in designing a rational strategy for the control of pest attack.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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AUTHOR CONTRIBUTION

MSK and SPG are responsible for the main concept and final approval of the manuscript. MVP helped in manuscript writing. AAS designed, performed, and coordinated the main study.

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