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Genetic variation of mitochondrial DNA in South Indian populations of *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae)

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Abstract
Genomic DNA of 16 different populations of *P. gossypiella* from South India were analysed for studying genetic variation. Amplicons of about 800 bp of mitochondrial COI gene was used and populations of *P. gossypiella* from various geographic locations were established as two distinct groups. Genetic relationships between the populations were evaluated by generating matrix and phonetic dendrogram was generated (by UPGMA method) and in PCA, first two components accounted for 100% of the similarity and the populations were differentiated into different classes based on band sharing data. The highest genetic variation (0.058) was found between populations of Beed and Madhira followed by Raichur and Guntur (0.055); the genetic variation was nil between the populations *viz.,* Adilabad and Anantapur, Aurangabad-2 and Aurangabad-1, Coimbatore & Aurangabad-1, Coimbatore & Aurangabad-2, Parbhani and B'gudi, Siruguppa and Aurnagabad-1, Siruguppa and Aurangabad-2, Siruguppa and Coimbatore, Siruguppa and Haveri, Siruguppa and Madhira populations.

Keywords: Population structure, *Pectinophora gossypiella,* genetic variation, mtDNA

1. Introduction
The pink bollworm, *Pectinophora gossypiella* (Saunders), is one of the most serious cotton pests in the world [3]. *P. gossypiella* was first described by Saunders in 1843 in India, where it was found damaging cotton. The origin of pink bollworm remains unknown, but the diversity of parasite species found in Pakistan [4] supports an Indo-Pakistan origin [10]. After the transport of cottonseed infested with diapausing larvae, *P. gossypiella* were carried over long distances to infest new areas. *P. gossypiella* was found in Mexico in 1916 and in Texas in 1917, from where it spread westward until it reached southern California in 1965 [15]. Spread of the pink bollworm through Sri Lanka was documented followed by Burma, Malaysia [11], and China before 1918 [9]. Presently, this invasive species has successfully established in many countries around the world and is considered a major pest in most cotton producing areas.
Despite the serious damage caused by *P. gossypiella* around the world, few studies have assessed genetic diversity and population structure, which are vital for pest management. In an earlier effort using allozyme electrophoresis, little genetic divergence was detected between a laboratory strain and wild strain [2]. Although microsatellite sequences are usually the most revealing DNA markers available for inferring population-genetic structure and dynamics, mtDNA is commonly used for investigating evolutionary history. Keeping this in view present study aimed to study the genetic plasticity among different locations of South India.

2. Material and Methods
Cotton bolls were collected from cotton growing states of Karnataka, Maharashtra, Andhra Pradesh, Tamil Nadu, Gujarat and Punjab at peak infestation levels in 2013-14 and 2014-15 seasons. From each state three districts were selected for collection. One of the main considerations in determining the sampling location was to represent all cotton eco-systems of South India. Thus, collected bolls were cut open and pink bollworm larval population was maintained in the laboratory for further studies. About 20 field collected healthy well grown 5th instar larvae were randomly collected in 70% alcohol from each location. Samples were brought to laboratory and kept at -20 ^{0}C until the isolation of DNA was done. At the time of isolation of total DNA, each larva was dissected and the gut contents were completely

removed to avoid any contamination of DNA by the food material it eats. Resulting skin and legs were used to prepare total DNA following procedure described by with some modification [5].

The larvae stored in alcohol were removed and kept on tissue paper for one minute. The larvae was dipped in liquid nitrogen and immediately transferred in to 1.5 ml micro centrifuge tubes. The larvae were grinded individually using micro pestle in micro centrifuge tubes containing 100 µl of preheated C-TAB extraction buffer and incubated at 65 ^{0}C for 30–45 minutes with occasional mixing. After incubation, the tubes were cooled to room temperature and equal volume of chloroform: isoamyl alcohol mixture (24:1) was added and mixed by inversion for 15 minutes. The suspension was centrifuged at 8000 rpm for 30 minutes at 4 ^{0}C. The supernatant was transferred to a fresh tube and equal (0.7 ml) volume of ice-cold isopropanol was added and mixed gently by inversion and kept at -20 ^{0}C overnight for DNA precipitation. The clear aqueous phase was transferred to a new micro centrifuge tube and the DNA pellet was separated from aqueous phase by brief centrifugation and the pellet was air-dried. Then, the DNA was dissolved in 100-150 µl of $T_{10}E_1$ (10mM Tris 1mM EDTA) buffer [21].

2.1 Purification of Genomic DNA

The genomic DNA isolated was purified according to the protocol described by [13]. To eliminate contaminating RNA, RNase (10 µl /100 µl) was added to DNA and incubated at 37 ^{0}C for 30 min by adding equal volume of chloroform: isoamyl alcohol (100 µl) and mixed thoroughly by repeated inversions. The mixture was centrifuged at 8000 rpm for 10 minutes at 4 ^{0}C, and the aqueous phase was transferred to another micro centrifuge to which two volumes of absolute alcohol was added and incubated at -20 ^{0}C overnight. DNA was pelleted by brief centrifugation and the supernatant was discarded. The pellet was washed with 70% ethanol and centrifuged at 8000 rpm for 5 minutes at 4 ^{0}C, the alcohol was discarded and the DNA pellet was air-dried completely. Depending upon the size of the pellet, DNA was dissolved in 25-50 µl of Tris-EDTA and stored at 4 ^{0}C. In order to make a better representation of each location equal amount of DNA from each of 5 larvae for each location was pooled and the resulting 14 bulked DNA samples were used for PCR analysis. Bulked DNA was diluted to 20-40 ng/ µl before actually being used in PCR reaction. The concentration of DNA was measured using NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE), and the quality was checked by 0.8% agarose gel electrophoresis before being used as template in PCR.

2.2 Quantification of genomic DNA

The genomic DNA isolated was quantified by ethidium bromide fluorescent quantification method described by [13]. The band intensity of isolated DNA in agarose dissolved in 40 ml of 1X TAE buffer containing ethidium bromide @ 0.5 µg per ml was compared with the band intensity of reference λ DNA Hind III digest. The quality and concentration of DNA was assessed by a spectrophotometer and also by gel electrophoresis using 0.8 per cent agarose with known concentrations of uncut λ DNA.

$$\text{DNA } (\mu g/\mu l) = \frac{OD_{260} \times 50 \text{ (dilution factor)} \times 50 \ \mu l/ml}{1000}$$

OD_{260}/ OD_{280} ratio was used to assess the purity of DNA. A ratio of 1.6 or less indicates that there may be proteins and/or other UV absorbers in the sample whereas ratio higher than 2.0 indicates sample may be contaminated with chloroform or phenol.

2.3 PCR amplification

The mitochondrial cytochrome oxidase subunit-I gene (800 bp) was amplified from 16 populations of *P. gossypiella* using primer pair: (5′TTGATTTTTTGGTCATCCAGAAGT-3′) (5′- TCCAATGCACTAATCTGCCATATTA-3′) as described by [17]. Reactions of 30-µl total volume contained 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mm MgCl2, 200 mol dNTP, 0.2 mol of each primer, 1 U of Taq DNA polymerase (M/S Bangalore Genei Pvt. Ltd., Bangalore), and 50 to 100 ng genomic DNA. Touchdown thermal cycling programs encompassing a 5 ^{0}C span of annealing temperatures at 55-50 ^{0}C were also used for the amplification in a Thermal Cycler (Bio-Rad, Hercules, CA). After an initial denaturation at 94 ^{0}C for 4 min, cycling parameters were 10 cycles of 94 ^{0}C for 20 seconds, highest annealing temperature (decreased 0.5 ^{0}C per cycle) for 30 seconds, and 72 ^{0}C for 30 seconds; and 30 cycles of 94 ^{0}C for 20 seconds, 50 ^{0}C for 30 seconds, and 72 ^{0}C for 30 seconds. This was followed by an extension step at 72 ^{0}C for 5 minutes.

Electrophoresis of PCR products was performed in 1.5% agarose gel containing ethydium bromide solution (10 mg/ml) along with 100 bp DNA ladder and amplicon products were measured using gel documentation unit. Following PCR amplification, the resulting amplicons of about 800 bp of mitochondrial *COI* gene were purified using the Spin Column PCR Product Purification Kit according to manufacturer's specifications. The purified PCR products were then inserted into the pGEM-T Easy Vector Systems (Promega) and transformed into competent *E. coli* strain DH5α by following manufactures instructions. Plasmid DNA was isolated and the presence of the insert was confirmed by restriction digestion of plasmid DNA with *Eco* RI restriction enzymes (Fermentas Life Sciences, Canada). Clones of interest were bi-directionally sequenced with the amplification primers commercially (Xcelris Labs Ltd, Ahmedabad, India). All the sequences were confirmed with NCBI BLAST database for the identity of the isolates based on previously published database sequences. Online multiple sequence alignment tools (www.genome.jp/tools/clustalw) were used to construct the phylogenetic tree using UPGMA-NJ method.

The evolutionary history was inferred using the Neighbor-Joining method [14] and the optimal tree with the sum of branch length = 0.08093345 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [6]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 16 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 210 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6 [18].

3. Results and Discussion

Populations of *P. gossypiella* collected from various geographic locations were established as two distinct groups (Table 1; Fig. 1). In PCA (Principle component analysis), the first two components accounted for 100% of the similarity. The first group (A) comprised 14 populations while the

distinct second group (B) consisted of only two populations. On the basis of simple matching coefficient, all the selected populations were grouped into five clusters. Cluster 1 had maximum of six populations followed by cluster 2 with four populations, cluster 3 with three cluster 4 with a single population and cluster 5 with two populations. The highest genetic variation (0.058) was found between populations of Beed and Madhira followed by Raichur and Guntur (0.055); the genetic variation was nil between the populations *viz.*, Adilabad and Anantapur, Aurangabad-2 and Aurangabad-1, Coimbatore & Aurangabad-1, Coimbatore & Aurangabad-2, Parbhani and B'gudi, Siruguppa and Aurnagabad-1, Siruguppa and Aurangabad-2, Siruguppa and Coimbatore, Siruguppa and Haveri, Siruguppa and Madhira, Siruguppa and Mysore, Siruguppa and Nalgonda populations.

The A group was subdivided into A1 and A2 at a coefficient value of 0.011, where A2 comprised of only one population from Guntur. The A1 group comprising thirteen geographical populations included three distinct groups (at a coefficient value of 0.009) A1.11, A1.12 and A1.2; A1.11 group consisting of Aurangabad-2, Siruguppa, Coimbatore, Aurangabad-1, Haveri and nanded, A1.12 group consisting of Mysore, Nalgonda, B'gudi and Parbhani while A1.2 comprised of Madhira, Adilabad and Anantapur population. Group B comprised of two populations Raichur and Beed at a coefficient value of 0.005.

The present study revealed very low level of genetic variation among *P. gossypiella* populations. The coefficient values varied from 0.000 to 0.058 across populations. Significant genetic similarity was found among populations in close proximity, as well as among those from distant parts of the range. On a larger scale, genetic differences among populations appeared to result from low dispersal rates between populations. The genetic variation detected in present study, among populations, was on lower side (0.000 to 0.058). As an invasive species, *P. gossypiella* might have undergone founder effects and suffered severe genetic drift in the past resulting in a loss of genetic diversity. The number of introductions, the size of each introduction, and subsequent drift and selection pressures that occur after introduction all can affect the amount of haplotypes and genetic variation in invasive populations [14]. Low mitochondrial DNA variation has been repeatedly reported in taxa that have undergone severe bottlenecks or founder effects [8, 12, 16]. Therefore, the *P. gossypiella* populations from south India that we studied are likely to have each undergone bottlenecks, as suggested by the low nucleotide and amino acid diversity observed (Fig. 2 and 3).

Extremely low genetic variation was observed in the Chinese population of Pectinophora gossypiella in the two mitochondrial regions among all populations examined. The low level of population genetic variation of *P. gossypiella* is attributed to invasion bottlenecks, which may have been subsequently strengthened by its non-migratory biology and the mosaic pattern of agricultural activities [22].

The pine processionary moth *Thaumetopoea wilkinsoni* showed a reduction in genetic variability at both mitochondrial COI and COII regions at the expanding boundary of the range, founded by few individuals expanding from nearby localities [17]. The low haplotype and nucleotide diversities among Australian diamondback moth *Plutella xylostella* L. mtDNA suggest a relatively recent bottleneck in population size [16].

Compared with *H. armigera*, which can make long-distance migratory movement [20]; *P. gossypiella* has a narrow host range and limited dispersal ability [7, 19]. Therefore, there should be limited gene flow among populations, and local populations are liable to be suppressed greatly, which might cause the population genetic variation to decrease, mtDNA is maternally inherited and more prone to genetic drift than nuclear markers because of the smaller effective population size and uniparental (maternal) gene flow [1, 8].

Table 1: Co-efficient values of geographic populations of Pink bollworm.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Pg1																
Pg2	0.000															
Pg3	0.017	0.017														
Pg4	0.017	0.017	0.000													
Pg5	0.024	0.024	0.012	0.012												
Pg6	0.017	0.017	0.000	0.000	0.012											
Pg7	0.034	0.033	0.020	0.020	0.026	0.020										
Pg8	0.018	0.018	0.006	0.006	0.012	0.006	0.020									
Pg9	0.017	0.017	0.033	0.033	0.012	0.033	0.020	0.006								
Pg10	0.017	0.017	0.022	0.022	0.012	0.045	0.020	0.006	0.033							
Pg11	0.017	0.017	0.016	0.016	0.012	0.028	0.020	0.006	0.033	0.011						
Pg12	0.019	0.019	0.060	0.006	0.019	0.006	0.026	0.006	0.006	0.006	0.006					
Pg13	0.020	0.020	0.070	0.007	0.000	0.007	0.020	0.007	0.007	0.007	0.007	0.013				
Pg14	0.038	0.044	0.031	0.031	0.044	0.031	0.055	0.031	0.003	0.031	0.031	0.038	0.041			
Pg15	0.012	0.012	0.000	0.000	0.012	0.000	0.020	0.000	0.000	0.000	0.000	0.006	0.007	0.031		
Pg16	0.035	0.035	0.008	0.008	0.018	0.008	0.043	0.018	0.058	0.041	0.033	0.010	0.011	0.009	0.009	

*Pg- *Pectinophora gossypiella*

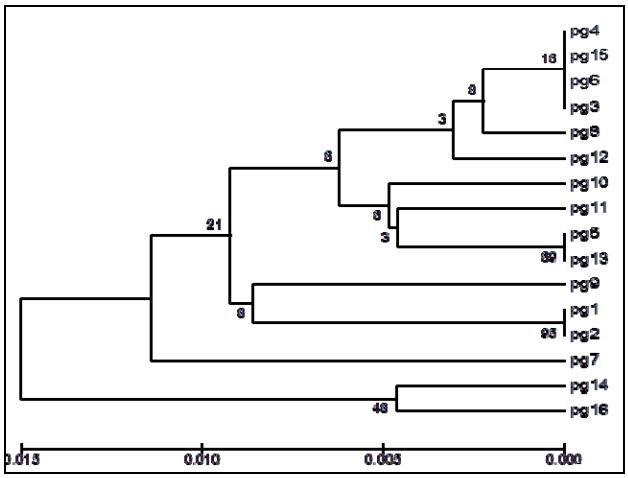

Fig 1: Dendrogram constructed based on coefficients with UPGMA-SAHN clustering method among 16 populations of *P. gossypiella* using multiple sequences alignment tools. Pg1: Adilabad; Pg2: Anantapur; Pg3: Aurangabad-1; Pg4: Aurangabad-2; Pg5: B'gudi; Pg6: Coimbatore; Pg7: Guntur; Pg8: Haveri; Pg9: Madhira; Pg10: Mysore; Pg11: Nalgonda; Pg12: Nanded; Pg13: Parbhani; Pg14: Raichur; Pg15: Siruguppa; Pg16: Beed.

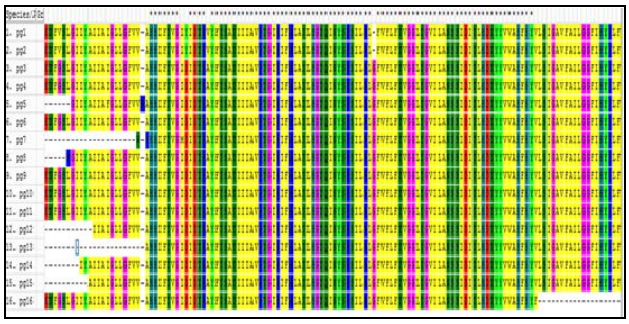

Fig 2: Amino acid alignment of 16 populations of *P. gossypiella*

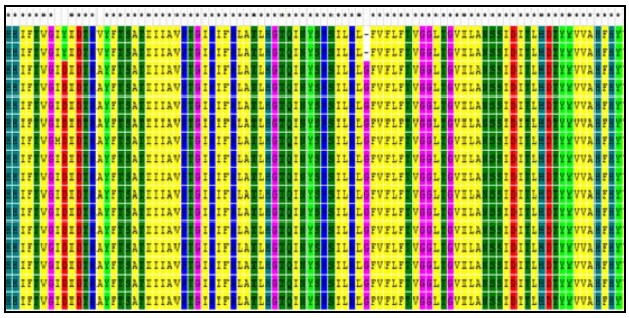

Fig 3: Conserved domain of CO-I in 16 geographic populations of *P. gossypiella*

4. Conclusion

On a larger scale, genetic differences among populations appeared to result from low dispersal rates between populations. The genetic variation detected in the present study, among populations, was on lower side (0.000 to 0.058). The genetic diversity across the different geographic locations revealed very low level of genetic variation among *P. gossypiella* populations.

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