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Population genetic structure of cotton pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) from India

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ABSTRACT

Pink boll worm, Pectinophora gossypiella became serious pest on BG II cotton hybrids globally causing huge economic losses in cotton even during later stages of crop growth. Understanding the genetic background and diversity of insect pests can aid in understanding their evolution in changing environments, hence aiding in effecting their management in an agricultural ecosystem. In the present investigation, the population genetic structure, distribution, and genetic diversity of P.gossypiella in cotton growing zones of India using the partial mitochondrial DNA cytochrome oxidase-I (COI) gene was addressed. Results revealed a total of 9 haplotypes (17.64%) identified from 51 individual sequences distributed in 16 populations belonging to different cotton growing zones of India. Diversity analysis of COI sequences revealed low genetic diversity (0.000 to 0.01066), high haplotype diversity (0.800), low nucleotide diversity (0.002), negative Tajima D (-0.670) values and high gene flow. This data on population genetics indicate populations of pink bollworm are genetically similar and Hap5, as an ancestral haplotype from which other haplotypes have evolved suggests that the migration and dispersal over long distance and invasiveness are major factors.

Keywords: *Genetic Variation, Mitochondrial DNA, Pink Bollworm, gene flow*

INTRODUCTION

Cotton (*Gossypium* spp.), the king of natural fibre, is being grown in 111 countries as commercial crop. India occupies about 25 per cent of global cotton area contributing to 12 per cent of world production. In India, cotton is cultivated in 125.84 lakh ha with a production of 360.0 lakh bales of seed cotton. The average productivity of cotton in India is 486 kg per ha. Despite the large area, productivity in India is said to be very low because the spectrum of insect pests on cotton is quite complex and as many as 1326 species have been listed on this crop across the world. However, 130 different species of insects and mites are reported to cause damage to cotton crops in India [1]. Among these, the bollworms *viz.,* American bollworm, *Helicoverpa armigera* (Hubner), spiny bollworm, *Earias insulana* (Boisdual), spotted bollworm, *Earias vitella* (Fabricius), pink bollworm, *Pectinophora gossypiella* (Saunders) pose greater threat to cotton production.

To avoid damage caused by bollworms, scientists have developed genetically modified hybrid cotton involving artificial insertion of a gene from the predominantly cultivated pest tolerant hybrid cotton containing only one gene, Cry1Ac gene has led to the management of all bollworms *viz.*, *H. armigera*, *E. vitella* and *P. gossypiella* successfully [2]. To retain the sustainability of Bt cotton and delay resistance in bollworms to BGI, second generation Bt cotton (BG II) expressing two Cry toxins (Cry1Ac+Cry2Ab2) was commercialized in USA (2003) and India (2006) and replaced >95 per cent of conventional cotton cultivation.

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Recently pink boll worm, *P.gossypiella* became serious pest on BG II cotton hybrids which became economically the most destructive insect pest of cotton. The main attributes for this issue include non-compliance of refuge, too many Bt hybrids of different durations, mono-cropping with extension of the crop season, lack of proper monitoring of bollworm and its resistance to gene and congenial climate [5]. In addition, reports on development of resistance in laboratory strains or field evolved resistance to *Cry1Ac* [6] and *Cry2AB* [7] or both the genes. Pink bollworm inhabiting distinct localities can experience different ecological and climatic conditions giving rise to variation in one or more traits. According to [14] , genetic structure and genetic diversity of the population play important role in defining the level of susceptibility of a population to any environmental changes. The selection pressure in the changed environment [13] as well as population structure, connectivity and genetic diversity define the level of susceptibility of a population and its adaptive capacity to environmental changes. Gene flow, through dispersal and short or long distance migration, plays a role in determining genetic variation and evolution of local populations [8]. Mitochondrial DNA is commonly used for investigating evolutionary history. As India is home to extraordinary variety of climatic conditions, extensive availability of host plants, and alternate host plants there is a necessary to understand the genetic variability of the species, which would help to develop management strategies.

MATERIALS AND METHODS

Pink bollworm infested cotton bolls were collected from different cotton growing districts of Telangana and other cotton growing states of India *viz.,* Andhra Pradesh, Karnataka, Maharashtra, Gujarat and Punjab in 2019-20 from November to January. Table.1 represented cotton growing zones of Telangana and some parts of India. Infested cotton bolls were cut open and separated larvae were used for DNA isolation for estimation of

genetic variability**.** Fourth instar larvae were surface sterilized in ethanol and individual larva was transferred into 1.5 ml eppendroff tube and stored in -80°C

Individual larvae were transferred to micro centrifuge tubes and the tubes were dipped in liquid nitrogen for one minute later larvae were ground with a sterile pestle to a fine powder with the help of homogeniser immediately after removing from the ultra freezer (-80ºC). DNA isolation was done by following the standard procedure mentioned in the macherey nagel NucleoSpin® Tissue kit. The genomic DNA obtained was checked for its quality on agarose gel. The gel (0.8%) was prepared by dissolving .04g agarose in 1x TAE buffer and boiling. About 2 µl of ethidium bromide (EtBr) was added to 50 ml of the agarose and poured in the electrophoresis tray placed with a comb and allowed to solidify for around 20-30min. Thereafter, it was placed in the gel electrophoresis unit with a running buffer. The DNA samples were loaded on the gel and electrophoresis is carried out @5v/cm of gel. The gel was imaged using gel documentation system (Alpha Innotech, USA).

PCR amplification and sequencing

The CO1 gene amplification was carried out for the DNA samples using primers 5´TTGATTTTTTGGTCATCCAGAAGT3´and 5´TCCAATGCACTAATCTGCCATATTA3´ with the master mix of Total reaction volume - 50 µl contained 2.5pm/ µl Forward primer - 5 µl, 2.5pm/ µl Reverse primer - 5 µl, Template DNA(50ng/ µl) - 5µl, 10X PCR buffer - 5 µl, 2.5mM dNTPs - 5 µl, Proofreading Taq polymerase (5U/ µl) – 0.5 µl, Water – 24.5 µl. PCR was performed using an Initial denaturation at 94 ºC for 5 min followed by 35 cycles of 94 ºC for 1 min, 50 ºC for 30 min, 72 ºC for 1 min and a final extension of 72 ºC for 7 min then Hold at 4°C. Following PCR amplification, the products are separated on 1% agarose gel. Then resulting amplicons were purified using the Spin Column PCR Product Purification Kit.

Sequencing of amplified samples and submission of sequences

The amplified sequences were sent for sequencing to bioserve biotechnologies Pvt. Ltd., Hyderabad, for sequencing and the nucleotides were retrieved from the trace files provided. The sequences were analysed in NCBI-BLAST (Basic Local Alignment Search Tool) to check for homology and annotated. The sequences were then submitted to GenBank and the accession numbers were received (MT362465-MT362480).

Data analysis

A total of 16 populations were selected to study population genetics of pink bollworm. Genomic DNA of three individuals from each population was chosen for population studies. *COI* sequence obtained from both strands in FASTA format were aligned using CLUSTALW programme [19] implemented in MEGA 7.0 software package sequence alignment application [9]. The sequences with missing nucleotides were not considered. The identity of all the sequences was confirmed with the Basic Local Alignment Search Tool (BLAST) of National Centre for Biotechnology Information (NCBI). The phylogenetic relationship among the populations was inferred with maximum likelyhood method using MEGA7 and pair wise distances was used to compare between sets of variables. Number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (Pi), polymorphic sites (S), Tajima's D tests of neutrality were obtained using DnaSP 6.1 software [15]. Evolutionary and geographical relationships among haplotypes were depicted by a median joining (MJ) method. Haplotype network was constructed by using NETWORK 4.6.1.1 software [3]. Genetic variation among the populations was measured by GST. Which was calculated by subtracting heterozygosity of a single population (Hsingle) from the heterozygosity of the total population (Htotal) and then by dividing by the heterozygosity of the total population (GST = (H total-H single)/H total).

Gene flow among pink bollworm populations (N*m*) was estimated by using the corresponding GST as: N*m* = 0.5(1-GST)/GST [11], where N is the number of individuals in a population and *m* is the proportion of those individuals resulting from immigration [20]. Generally Nm values >1 indicate the presence of significant gene flow among the populations. GST and gene flow were calculated using DnaSP 5.1.

RESULTS AND DISCUSSION

Genetic variability among *P. gossypiella* populations using mitochondrial Cytochrome C Oxidase gene (COI)

Fifty one pink bollworm individuals from 16 populations were sequenced for partial mitochondrial COI region to workout the population genetic structure, genetic diversity

and distribution in three cotton-growing zones of India. The nucleotide sequences of 748 bp were aligned using CLUSTEW and edited using MEGA7 to ensure no missing data for all samples. The insertions or deletions were checked and corrected in all the sequences. Then, the sequences were BLAST analyzed in NCBI and found consensus with the PBW sequences from various parts of the world.

Phylogenetic tree was constructed with sixteen populations of *P. gossypiella* applying Maximum likelihood algorithm to JTT matrix-based model using MEGAX. Cluster diagram grouped all 16 populations in to three major clades (Figure 1). Clade I includes a total of 10 populations, of which 9 are from south India (Rangareedy, Karimnagar, Warangal, Nalgonda, Guntur, Kadapa, Kurnool, Chennai and Bangalore) and one from north India (Faridkot). Clade II includes 5 populations of which three from central India (Buldhana, Akola, Junaghar) and two are from south India (Raichur and Adilabad) whereas clade III includes only one population from Mahabubnagar.

Pair wise distances

Genetic distance among the populations of *P. gossypiella* was calculated using pair-wise distances analyzed by poisson correction model and gave values ranging from 0.000 to 0.01066 (Table 2). This suggested that there is no much variation among the population of *P. gossypiella* in India. The highest genetic distance (0.01066) was observed between populations of Mahabubnagar with Raichur. The genetic distance was nil among the populations viz., Bangalore, kadapa, Karimnagar, Guntur, punjab, Ranga Rededy, Warangal, Kurnool and Tamil Nadu of clade I and Buldhana, Adilabad, Akola and Gujarat of clade II. The present study revealed a very low level of genetic variation among *P. gossypiella* populations as the genetic distance varied from 0.000 to 0.01066 across populations. On a larger scale, low genetic differences among populations appeared to result from low dispersal rates between populations.

Nucleotide polymorphism

The nucleotide composition analysis of 51 sequences showed nucleotide frequencies as A= 31.56%, T = 41.16%, C= 14.42 %, G= 12.83% and was found to be A+T rich (72.72%). A+T rich

nature of nucleotide sequences of COI in *P. gossypiella* was similar to many reported species. The sequence region showed high AT rich nucleotide composition, which is a typical characteristic of mitochondrial genes. The estimated transition to transversion ratio bias (R) was 1.50. Monomorphic sites (Invariable) were 740 and a total of 8 polymorphic sites were observed, with number of mutations 8 which including parsimony informative sites (two variants): 8(site positions: 125, 261, 500, 659, 673, 678, 696, 726).

Diversity indices

Eight polymorphic sites were found among 51 individuals of *P. gossypiella* from 16 populations. Demographic statistics like number of haplotypes (n), haplotype diversity (Hd) and nucleotide diversity (n) are given in (Table 3). haplotype diversity and nucleotide diversity among the populations ranged from 0.000 to 0.800 and 0.000 to 0.002, respectively. The average haplotype diversity (Hd) and nucleotide diversity (n) of COI gene were 0.763 and 0.0017 respectively. Average number of nucleotide differences (k) found to be 1.340. Haplotype diversity and nucleotide diversity indices were high in Karnataka (0.800 ± 0.029; 0.002) followed by Telangana (0.725 ± 0.27; 0.0088) and lowest (0.000 ± 0.00; 0.000) in Maharashtra, Gujarat, Punjab and Tamil Nadu populations. When these populations were pooled zone wise, South Indian population showed high haplotype diversity (0.764) and nucleotide diversity (0.0019) followed by central India (0.181; 0.0002).

Haplotype, Network and Demographic analysis

Sequence analysis of 51 individuals of *P.gossypiella* from 16 locations yielded 9 haplotypes (Table 4). Among all haplotypes, Hap5 was observed to be the dominant one shared by 20 sequences represented by samples from 7 locations of three southern states of India and one northern state. Hap4 sharing 13 sequences represented from 4 locations (3 from central India and 1 of south India). Whereas Hap3 is shared by 8 locations, however, Hap1 and Hap2 are shared by 3 locations each and Hap6 to Hap9 are shared by one location each. Out of 9 haplotypes, six were found in Telangana region, two in Andhra Pradesh, Karnataka and one in each of the other states.

Median joining network among haplotype.

Median joining haplotype Network constructed based on the 51 sequences of *P. gossypiella* populations of India identified 9 haplotypes (Figure 2). Network analysis indicated that Hap5 (39.21%) is present predominantly in southern states (Telangana, Andhra Pradesh, Karnataka) where 50% of analyzed populations of Telangana come under Hap5. Hap4 (25.49%) present in 2 states is shared by 3 populations of central India *i.e.,* Maharashtra (2) and Gujarat (1) and one population of Telangana (Adilabad) as it is adjoining district to Maharashtra state. MJ network suggests that the most common, Hap5 could be proposed as ancestral haplotype for the analyzed populations which occupied the central region of haplotype network connected with all other 8 haplotype

Tajima's D, test and Fu's Fs test

Neutrality indices like Tajima's D test and Fu's Fs test were calculated and have shown non significant negative values for overall average of all populations (Table 5). A negative Tajima's D signifies an excess of low frequency polymorphisms in the population consistent with the recent population expansion after bottleneck or selection sweep.

A positive Tajima's D signifies low levels of both low and high frequency polymorphisms. A negative value of Fu's Fs is evidence of an excess number of alleles, as would be expected from a recent population expansion or genetic hitchhiking. A positive value of Fu's Fs is evidence of a deficiency of alleles, as would be expected from a recent population bottleneck. Present results are similar to [17] who also reported that Tajima's D values were negative when grouped populations of India into both state-wise and zone- wise. The negative Tajima's D value indicates recent demographic expansion of the population. This shows that despite the distance between localities sampled there is no genetic structure among the *P. gossypiella* populations in India. Present results supports hypothesis of severe bottlenecks or founder effects in some of the taxa leading to low mitochondrial variations [16] [12] reported that insects under high insecticidal selection pressure undergo a genetic bottleneck and this may be true for pink bollworm as continuum of BG II in the south and central cotton zone of India is responsible for selection pressure.

Gene flow (Nm) between different populations of *Pectinophora gossypiella*

Nm values were calculated for 16 populations based on *COI* gene sequences. The results revealed that there is high, intermediate and low gene flow among the populations. High gene flow was recorded among populations of South India with North and Central India whereas low gene flow was recorded between North and Central India (Table 6). High rate of gene flow reduce genetic differentiation between the two groups with increasing homogeneity. This indicates that low genetic differentiation among the populations of *P.gossypiella* as depicted in the dendrogram

Present results were similar with findings of [4] who recorded a very low level of genetic variation among *P. gossypiella* populations of south India whose coefficient values varied from 0.000 to 0.058. [21] reported extremely low genetic variation in the Chinese population of Pink bollworm in the two mitochondrial regions. The low level of population genetic variation of *P. gossypiella* is attributed to invasion bottlenecks, which might have been subsequently strengthened by its non-migratory biology and the mosaic pattern of agricultural activities. The low haplotype and nucleotide diversities (mtDNA) among Australian diamondback moth, *Plutella xylostella* L. suggest a relatively recent bottleneck in population size [16]. Present findings are supported by [18] wherein they reported 12 haplotypes from 79 individual sequences to 19 *P. gossypiella* populations across India. Oncontrary to this [10] reported four haplotypes from *COII* and three haplotypes from *Nad4* region in 91 individuals of Chinese population

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CONCLUSION

The present study reports low genetic diversity (0.000 to 0.01066), high haplotype diversity (0.800), low nucleotide diversity (0.002), negative Tajima D (-0.670) values and high gene flow.

This data on population genetics indicates populations of pink bollworm are genetically similar which would be useful to develop area-wide management strategies like resistance management in the form of refugia requirement, development of resistant varieties, and dosage of pesticide to be used.

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Conflict of interest: The authors declare they have no conflict of interest.

Ethics approval and consent to participate: Not applicable.

Availability of data and materials: The genome sequence data supporting this study's findings are openly available in GenBank of NCBI at https://submit.ncbi.nlm.nih.gov/subs/?search= SUB7307262 with the accession number MT362465 to Mt362480.

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Table 1. Details of Pectinophora gossypiella sampled for genetic variability studies during 2019-2020.

S.No	Zone	Location		Date of Sampling	Geographical Coordinates	Population Code
		State	Place			
1.	South India	Telangana	Adilabad	24- Nov-2019	19.6808 N 78.5359 E	ADB
2.			Warangal	30-Nov-2019	17.7919 N 79.7974 E	WGL
3.			Rangareddy	2-Dec-2019	17.2326 N 77.5048 E	RNG
4.			Mahaboobnagar	21-Nov-2019	16.4547 N 78.8365 E	MHB
5.			Karimnagar	30-Nov-2019	18.2618N 79.7434 E	KRM
6.			Nalgonda	29-Dec- 2019	17.323 N 79.1656 E	NAL
7.		Andhra Pradesh	Kurnool	28- Nov-2019	15.7313 N 77.4356 E	KUN
8.			Kadapa	22- Nov-2019	14.416 N 78.23333 E	KAD
9.			Guntur	20-Jan-2020	16.3067 N 80.4365 E	GNT
10.		Karnataka	Raichur	29-Nov-2019	16.2120 N 77.3439 E	RAI
11.			Bangalore	7-Jan-2020	13.0270N 77.5843E	BAN
12.		Tamil Nadu	Chennai	10-Jan-2020	11.0152N 76.9326E	CHE
13.	Central India	Maharashtra	Akola	26- Nov-2019	20.6962 N 77.0589 E	AKL
14.			Buldhana	7-Jan-2020	19.2644 N 76.6413 E	PRB
15.		Gujarat	Anand	22-Nov -2019	22.5608 N 72.9547 E	GJT
16.	North India	Punjab	Faridkot	30-Nov-2019	29.9038 N 73.8771 E	PUN

Table 2. Pair wise distance of P. gossypiella populations.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
RAI	0.00000															
NAL	0.00370	0.00000														
KRL	0.00492	0.00205	0.00000													
BUL	0.00164	0.00204	0.00328	0.00000												
BNG	0.00328	0.00041	0.00164	0.00164	0.00000											
KDP	0.00328	0.00041	0.00164	0.00164	0.00000	0.00000										
MBNR	0.01066	0.00780	0.00900	0.00898	0.00737	0.00737	0.00000									
KRM	0.00328	0.00041	0.00164	0.00164	0.00000	0.00000	0.00737	0.00000								
CHE	0.00492	0.00205	0.00000	0.00328	0.00164	0.00164	0.00900	0.00164	0.00000							
ADB	0.00164	0.00204	0.00328	0.00000	0.00164	0.00164	0.00898	0.00164	0.00328	0.00000						
AKL	0.00164	0.00204	0.00328	0.00000	0.00164	0.00164	0.00898	0.00164	0.00328	0.00000	0.00000					
GNL	0.00328	0.00041	0.00164	0.00164	0.00000	0.00000	0.00737	0.00000	0.00164	0.00164	0.00164	0.00000				
JUN	0.00164	0.00204	0.00328	0.00000	0.00164	0.00164	0.00898	0.00164	0.00328	0.00000	0.00000	0.00164	0.00000			
FAR	0.00328	0.00041	0.00164	0.00164	0.00000	0.00000	0.00737	0.00000	0.00164	0.00164	0.00164	0.00000	0.00164	0		
R.R	0.00328	0.00041	0.00164	0.00164	0.00000	0.00000	0.00737	0.00000	0.00164	0.00164	0.00164	0.00000	0.00164	0	0	
WGL	0.00328	0.00041	0.00164	0.00164	0.00000	0.00000	0.00737	0.00000	0.00164	0.00164	0.00164	0.00000	0.00164	0	0	0

Table 3. Descriptive analysis for polymorphisms based on COI gene in Pectinophora gossypiella populations of India

Population	No .of Sequences	H	Haplotype diversity(Hd)	Nucleotide diversity(pi)	K	S
Telangana	18	6	0.725	0.002	1.510	5
Andhra Pradesh	9	2	0.500	0.0006	0.500	1
Karnataka	6	4	0.800	0.002	1.667	3
Maharashtra	6	1	0.000	0.000	0.000	0
Gujarat	4	1	0.000	0.000	0.000	0
Punjab	4	1	0.000	0.000	0.000	0
Tamil Nadu	4	1	0.000	0.000	0.000	0
South India	37	9	0.764	0.0019	1.477	8
Central India	10	2	0.181	0.0002	0.181	1
North India	4	1	0.000	0.000	0.000	0
Total	51	9	0.763	0.0017	1.340	8

H= Number of haplotypes, K= Average number of nucleotide differences,
S= Polymorphic sites

Table 4. Mitochondrial haplotype distribution of Pectinopora gossypiella in different populations.

	Telangana						Andhra Pradesh			Tamil Nadu	Karnataka		Punjab	Gujarat	Maharashtra	
Haplotypes	ADB	NAL	MBNR	RR	WGL	KRM	KNL	KDP	GNL	CHE	BNG	RAI	FAR	JUN	AKL	BUL
Hap1 (3)												3				
Hap2 (3)		3														
Hap3 (8)							3			4	1					
Hap4 (13)	3													4	3	3
Hap5 (20)				3	3	3		3	3		1		4			
Hap6 (1)			1													
Hap7 (1)											1					
Hap8 (1)			1													
Hap9 (1)			1													
Overall	3	3	3	3	3	3	3	3	3	4	3	3	4	4	3	3

Table 5. Tajima's D and Fu's F values of COI gene of Pectinophora gossypiella

Population	Tajimas'D	Significance	Fu's Fs
South India	-0.663	NS, P> 0.10	-2.958
Central India	-1.128	NS, P> 0.10	-0.410
North India	-	-	-
TOTAL	-0.670	NS, P> 0.10	-2.748

Table 6. Gene flow (Nm) between different populations of Pectinophora gossypiella based on COI region.

Population 1	Population2	Nm*	Gene flow
KN	TS	0.981	Intermediate
KN	AP	3.455	High
KN	MH	0.667	Intermediate
KN	GJ	0.828	Intermediate
KN	PUN	1.143	High
KN	TN	1.143	High
TS	AP	12.053	High
TS	MH	1.478	High
TS	GJ	1.874	High
TS	PUN	5.013	High
TS	TN	1.427	High
AP	MH	0.416	Intermediate
AP	GJ	0.534	Intermediate
AP	PUN	4.313	High
AP	TN	0.950	Intermediate
MH	GJ	0.000	Low
MH	PUN	0.000	Low
MH	TN	0.000	Low
GJ	PUN	0.000	Low
GJ	TN	0.000	Low
PUN	TN	0.000	Low
South India	North India	5.423	High
South India	Central India	1.616	High
North India	Central India	0.000	Low

**Nm >1 (High gene flow); Nm= 0.25-0.99 (Intermediate gene flow); Nm = < 0.25 (Low gene flow).*

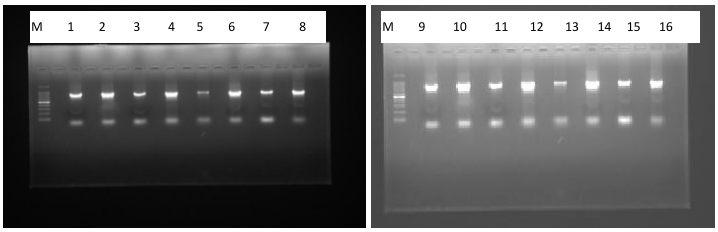

Plate 1. PCR amplified product of COI gene of 16 Pectinophora gossypiella populations of India. M= 100 bp Ladder, Lane 1= ADB, 2= WGL, 3= MBNR 4=RR,5=NAL, 6=KRM, 7=KNL, 8=KDP, 9=GNL, 10=BNG, 11=RAI, 12=AKL, 13= BUL, 14=JUN, 15=FRD, 16= CHE.

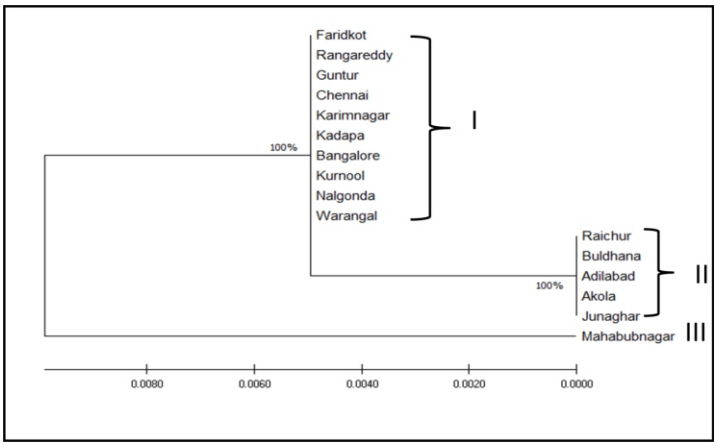

Figure 1. Maximum likelihood tree of CO1 sequences from 16 populations of P. gossypiella.

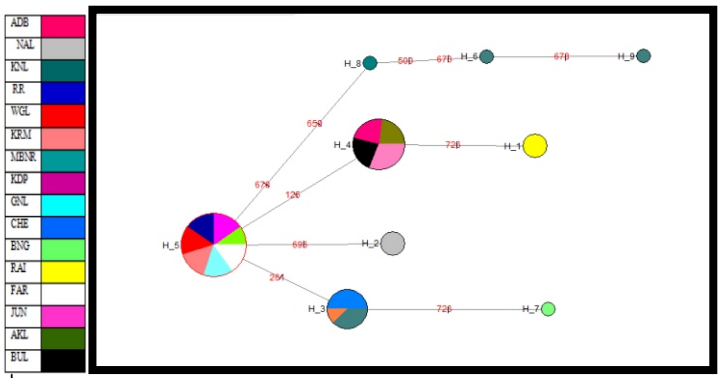

Figure 2. Median joining haplotype network based on COI sequences of Pectinophora gossypiella populations from India. The mutated positions between haplotypes are given in numerics.

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