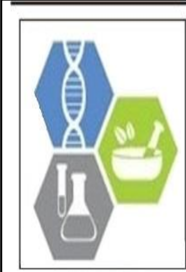


Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

J E Z S Journal of Entomology and Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2020; 8(1): 1682-1687

© 2020 JEZS
Received: 28-11-2019
Accepted: 29-12-2019

Shahanaz
Department of Entomology, College of Horticulture, Mojerla, SKLTSHU, Hyderabad, Telangana, India

Vinay K Kalia
Division of Entomology, ICAR-IARI, New Delhi, India

Corresponding Author:
Shahanaz
Department of Entomology, College of Horticulture, Mojerla, SKLTSHU, Hyderabad, Telangana, India

Phylogenetic studies in cotton pink bollworm (*Pectinophora gossypiella*) using *Actin* gene

Shahanaz and Vinay K Kalia

Abstract
The pink bollworm, *Pectinophora gossypiella* is one of the devastating pests of cotton and has become a major threat to Bt cotton growers across India since 2015. To study the possibility of any genetic variation among the 16 populations of *P. gossypiella*, experiments were conducted with a promising nuclear protein-encoding gene Actin (β ACT). The phylogenetic tree constructed depicts that there is low genetic variation among populations *of P. gossypiella*. A neighbor-joining tree constructed based on Tamura parameters of 3 β ACT gene sequences produced two clusters. The cluster diagram shows that a total of 16 populations grouped into two major clades. However, the *P. gossypiella* populations were separated by the *Spodoptera frugiperda* sequence, which shows a similarity of 91%.All populations were grouped into two major clades, each comprising 9 and 7. Negative value of Tajima's d indicates that population expansion or selection pressure on the population which lead to presence of an excess of low-frequency polymorphisms.

Keywords: Cotton pink bollworm, *pectinophora gossypiella*, *actin* gene

Introduction
Bt transgenic cotton was commercialized in India since 2002 and its rapid adoptability by the farmers has increased the income of the framers and reduced the pesticide consumption on the cotton. Earlier, American bollworm, *Helicoverpa armigera* was considered to be a key pest and many farmers committed suicide in India. However, it has attained secondary pest status whereas *P. gossypiella* has become major concern to the farmers and scientist as it attacks majorly cotton. The Adult female lays eggs on flower buds, calyx, bolls, upon hatching larvae enter the bolls and feeds on the seed lint. Since economic part in cotton is fiber and seed oil and pink bollworm incidence during later stage of the crops causes yield losses to an extent of 4 to 5 q/ha.
In some localities of Gujarat of North India, it was observed that development of resistance to Bt transgenic cotton (Bollgard-I) containing Cry1Ac toxin by pink bollworm. (Dhurua and Gujar, 2011) [1]. Since 2015 onwards pink bollworm damage to Bollgard-II containing two toxins (Cry1Ac + Cry2Ab) was observed in Telangana, Maharashtra, Andhra Pradesh, Gujarat in Gujarat (Naik *et al.,* 2018) [2].
To understand and develop effective integrated pest management strategies studies on genetic diversity in pink bollworm using different markers is essential and few studies were carried out by using microsatellite markers (Liu *et al.,* 2008) [12] and mitochondrial cytochrome C oxidase (COX I) gene (Sridhar *et al.,* 2016).
Mitochondrial DNA (mtDNA) is generally thought to change faster than normal DNA (nDNA) and has been shown to have a different inheritance pattern (Behura 2006) [13]. Nucleoprotein-coding genes are good candidates because they are more efficient and more developed than mitochondrial-coding genes, have little length, flexible, and vary within and between genes (Friedlander *et al.,* 2017., 1992, 1994), but the problem with these genes is that they occur in small numbers. Recently, several protein-coding genes have become more widely used, among which elongation factor-1a (EF-1a) has been shown to be useful in linking species and subfamily relationships in Lepidoptera (Sutrisno *et al.,* 2006) [15]. Genetic diversity of *P. gossypiella* population has been identified in several regions of India using the EF-1 α gene and low genetic variation between populations has been reported (Shahanaz and Kalia VK). However, EF-1α, Actin genes have been found to be a useful gene as it codes amono acids and its conserved nature.
Keeping the above facts in view, this study was carried out using nuclear protein codig gene

to detect the population structure, genetic diversity, genetic differentiation among the *P. gossypiella* populations.

Materials and Methods
Sample Collection
Pink bollworm infested cotton bolls were collected from the sixteen different locations of India during 2016-17. Mature larvae of *P. gossypiella* were extracted from infested cotton bolls using forceps (Table 1). Among this five larvae from each location were selected, starved for more than 12 hours and surface sterilized with 70% ethanol. Later the sterilized larvae were transferred into the micro centrifuge tubes (MCT) stored at –80 °C to study phylogenetic analysis.

Table 1: List of *Pectinophora gossypiella* populations used for phylogenetic studies

S. No	Location		Date of Sampling	Geographical Coordinates	Population Code
	State	Place			
1	Telangana	Adilabad	24- Nov-2016	19.6808 N 78.5359 E	ADB
2		Warangal	26-Nov-2016	17.7919 N 79.7974 E	WGL
3	Andhra Pradesh	Macherla	28-Nov-2016	16.4773 N 79.4375 E	GNM
4		Lam	31-Dec-2015	16.3067 N 80.4365 E	GNL
5		Kurnool	23-Dec-2015	15.7313 N 77.4356 E	KNL
6		Anantapur	22-Dec-2015	14.6434 N 77.5996 E	ANP
7		Pamidi	22-Dec-2015	14.9512 N 77.5848 E	ANBP
8	Karnataka	Dharwad	23-Dec-2016	15.4461 N 74.9986 E	DWD
9		Raichur	28-Nov-2017	16.2120 N 77.3439 E	RCH
10	Maharashtra	Akola	12-Jan-2016	20.6962 N 77.0589 E	AKL
11		Aurangabad	07-Nov-2017	19.8762 N 75.3433E	AUB
12		Parbhani	16- Nov-2017	19.2644 N 76.6413 E	PBN
13	Gujarat	Anand	18-Dec-2016	22.5608 N 72.9547 E	GJA
14		Junagarh	19-Dec-2016	21.5221 N 70.4578 E	GJJ
15		Manglor	18-Dec-2016	21.1329 N 70.1154 E	GJM
16	Rajasthan	Sri Ganganagar	11-Nov-2016	29.9038 N 73.8771 E	GNG
17	Delhi	Lab (IARI)	30-Dec-2016	28.6448 N 77.2167 E	LAB
18		Field (IARI)	11-Dec-2015	28.6448 N 77.2167 E	NDL

DNA extraction, amplification and sequencing
Single healthy larvae from each location were collected to extract genomic DNA by method described by Gujar *et al.* (2007) with minor modifications. Individual larvae were removed from – 80 ºC deep freezer and immediately crushed into fine powder using a sterile pestle to avoid thawing of the larvae. To the homogenized larval sample pre-warmed DNA extraction buffer (2% Cetyl trimethyl ammonium bromide, 1.4 M NaCl, 100 mM Tris HCl, 20mM EDTA) 400 µl(at 60 ºC for 10 minutes) and 4 µl β-mercapto ethanol was added. Incubate the samples at 65 °C in dry bath for 1 h by shaking for every ten minutes interval. To this incubated sample, an equal volume of chloroform: isoamyl alcohol (24: 1v/v) was added, mixed by gentle inversion, then centrifuged at 12000 rpm for 10 minutes at 25 °C. After separating aqueous phase in to fresh tube, add 0.6 volume of Isopropanol and 150µl of 4M NaCl then gently mix them, incubate at room temperature for one hour to allow DNA to precipitate. Then centrifuge the sample at 10000 rpm for 10 minutes. The upper supernatant was removed and below DNA is settled in pellet form. Later wash the pellet twice with 70% ethanol and air dry for 15 minutes. Dissolve the DNA pellet in 50µl of nuclease free water. Incubate the DNA samples at 37 ºC for 1 h with addition of RNAase to the samples. Observe the extracted DNA by running on 0.8% agarose gel, store the DNA at -20º C.

Genetic variability of *P. gossypiella* populations were carried out using nuclear protein coding gene, β Actin (*βACT*), selected for diversity analysis. The DNA was amplified on PCR for 460 bp region by using in Gen Pro (Biover) Thermal Cycler. The primer sequences of nuclear protein coding gene, *βACT* were synthesized from the sequence of respective gene of *Helicoverpa armigera* available in Gen Bank as no accession of these genes were available for *P. gossypiella*. Eight pairs of (forward and reverse) primers for β Actin gene were custom synthesized by Eurofins Analytical Services India Pvt. Ltd, New Delhi, Delhi -20 and were used to amplify the *P. gossypiella βACT* (Table 2.).

The sample for PCR amplification mixture was prepared for 50 µl by taking 5.0 µl of 1X PCR Buffer (10 mM Tris/HCl, 50 mM KCl, 2 mM Mgcl2), 2 µl of 10 pmol of each primer, 2.5mM of 4 µl of each dNTP and Taq polymerase 1U and template DNA 2 µl. (PCR run for 40 cycles with initial denaturation at 94 °C for 5 min, denaturation for 1min at 94 °C, annealing for 1 min at 58 °C, extension for 2 min at 72 °C and a final extension at 72 °C for 10 min. The amplified products were resolved on 1.2% agarose gel and visualized in gel documentation system and were sequenced (Chromus Biotech Pvt. Ltd, Bengaluru, India).

Table 2: List of Primers of nuclear protein coding gene *βACT* used for initial screening of *P. gossypiella*

Gene*	Primer's Name	Sequence 5' to 3'
βACT 1F	Forward	CCCTCTATCGCCACTAGGAA
βACT 1R	Reverse	AATCGACAATGTTCCGCATT
βACT 2F	Forward	TGGTATTGCTGACCGTATGC
βACT 2R	Reverse	AAGCACTTCCTGTGGACGAT
βACT 3F	Forward	TGGTATTGCTGACCGTATGC
βACT 3R	Reverse	GCGCTTAGAAGCACTTCCTG
βACT 4F	Forward	TGGTATTGCTGACCGTATGC
βACT 4R	Reverse	GGGCCAGACTCATCGTACTC
βACT 5F	Forward	TGGTATTGCTGACCGTATGC
βACT 5R	Reverse	GGCCAGACTCATCGTACTCC
βACT 6F	Forward	TGCGTGACATCAAGGAGAAG
βACT 6R	Reverse	GTGTTGGCGTACAGGTCCTT
βACT 7F	Forward	AAGGCCAACAGGGAGAAGAT
βACT 7R	Reverse	ATGACACGGTTGGAGTAGCC
βACT 8F	Forward	AAGGCCAACAGGGAGAAGAT
βACT 8R	Reverse	AGGAAGGAAGGCTGGAAGAG

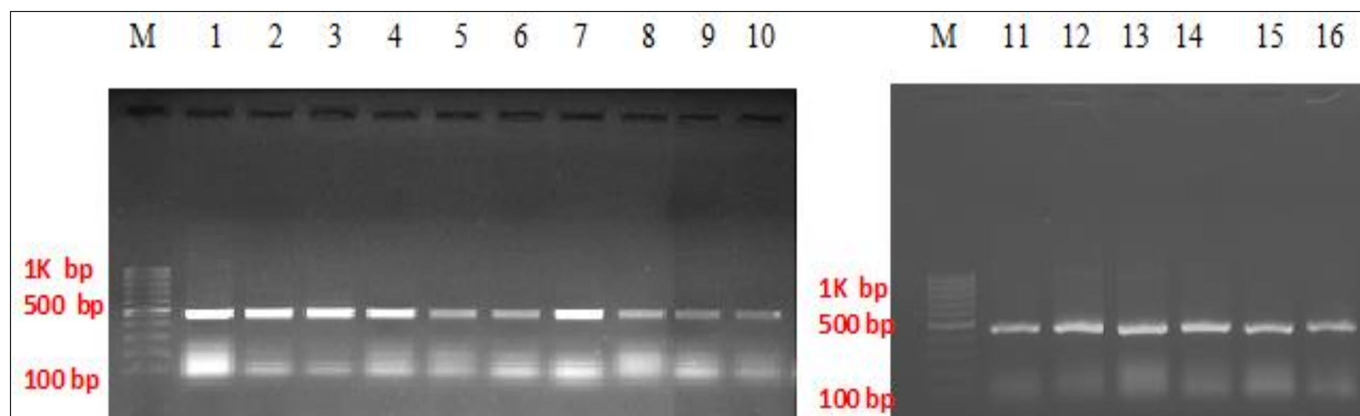
* Elongation Factor 1Alpha (*EF-1α*), β Actin (*βACT*) β tubulin (*βTUB*)

Phylogenetic Analysis

Best quality PCR products of 13-15 populations of *P. gossypiella* encoding *βActin* genes were selected and sent for sequencing. *βActin* sequences were aligned in ClustalW programme (Thompson *et al.,* 1994) [5] implemented in sequence alignment application MEGA 7.0 software package (Kumar *et al.,* 2016) [6]. Later, sequences were trimmed and aligned and subjected to Mutiple sequence alignment for diversity studies. Homology search of the final Actin sequences obtained were done in BLAST (Basic Local Alignment Search tool) of the NCBI (National Centre for Biotechnology Information) using non redundant nucleotide database and protein database in Balstn and Blastx searches. Total 16 sequences were deposited in GenBank to get GenBank accession numbers (Table 3).

Results and Discussions

Multiple alignment of sequences of sixteen populations of *P. gossypiella* were performed in CLUSTAL W of MEGA 7.0 version. Approximately 460 bp sequence length of Beta Actin (*βACT*) was obtained by using forward and reverse primers (Fig 1). Similarity search of the nucleotide sequence of the *βACT* gene performed in BLAST programme. The results indicate that *P. gossypiella* has a greatest identity with many of the of Lepidoptera insects species. *P. gossypiella βACT* sequence showing high identity with the *βACT* mRNA sequences of *Spodoptera frugiperda* (91% homology) available in the NCBI nucleotide database.



M= 100 bp Ladder, Lane 1= ADB, 2= WGL, 3=GNM, 4=GJM, 5=GJJ, 6=GJA, 7=KNL, 8=APN, 9=GNL, 10=NDL, 11=AKL, 12=ANBP, 13= DWD, 14=LAB, 15=GNG, 16= PBN

Fig 1: PCR amplified gel image of *βACT* gene of different Populations of *P. gossypiella*

Genetic divergence of the individuals were estimated for Tamura 3 parameter as number of base substitutions per site and graphically depicted by a neighbor joining tree using MEGA 7.0. Mean distance of population was 0.018 ±0.003. The analysis of nucleotide composition shows that sequences are rich in GC ratio with composition of nucleotides *viz*., A=19.13%, T=21.46%, C=34.94%, G=24.47%. The ratio of transition to trans version was (R=0.65). Overall nucleotide diversity (π) of the *βACT* sequences was 0.0147. Number of segregating sites (S=29) for *βACT* gene were used to calculate Tajima's D statistic value and it was -0.9123. The Tajima's D values are non-significant and negative which depicts that population has excess amount of polymorphism with low frequency. This is due to population might have under selection of size expansion.

Genetic distance values ranging from 0.000 to 0.038 among the *P. gossypiella* populations which is calculated with pair-wise Tamura3 parameters (Table 3). This suggested that not much variation in the populations of *P. gossypiella* in India. Highest genetic distance (0.038) was evidenced between Lab (LAB) and Adilabad (ADB) followed by, Lab and Guntur (GNM). However, lowest genetic distance (0.000) was observed between Warangal (WGL) and Junagarh (GJJ) followed by Dharwad (DWD) with Anantapur Non Bt(ANBT), Akola(AKL), Anand (GJA).

Phylogenetic tree constructed with sixteen populations of *P. gossypiella* applying Neighbor-joining algorithm to Tamura 3 parameter matrix using the Maximum Composite Likelihood (MLC) approach. Total branch length of phylogenetic tree was 0.0739 base substitutions per site. Cluster diagram reveals that total 16 populations grouped in to two one major clade (Fig 2, 3). However *P. gossypiella* populations were separated with the sequence of *Spodoptera frugiperda* which is showing similarity of 91%. All the populations were grouped into two major clades each consists of 9 and 7, respectively. Both the clades comprises of all the populations irrespective of geographical distribution. Major clade I consists of nine populations which includes two North Indian (LAB, GNG) along 2 Andhra Pradesh (KNL, GNL, GNM, APN), one Telangana (WGL) and Gujrat (GJJ, GJA). While, major clade II shared by 2 Maharashtra (AKL, PBN), DWD, NDL, ADB, GJM, and ANBP populations.

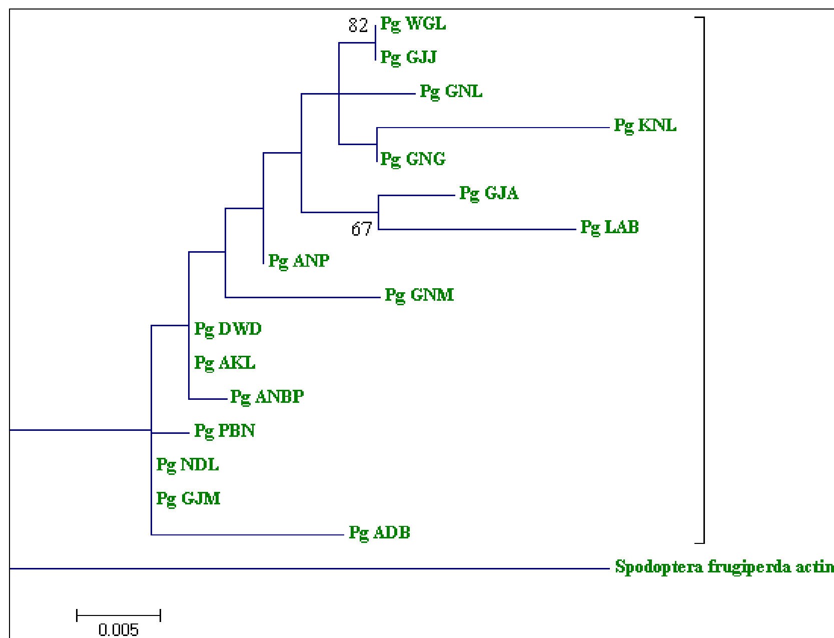


Fig 2: Neighbour joining tree constructed based on T3P parameter for 16 populations of *P. gossypiella*

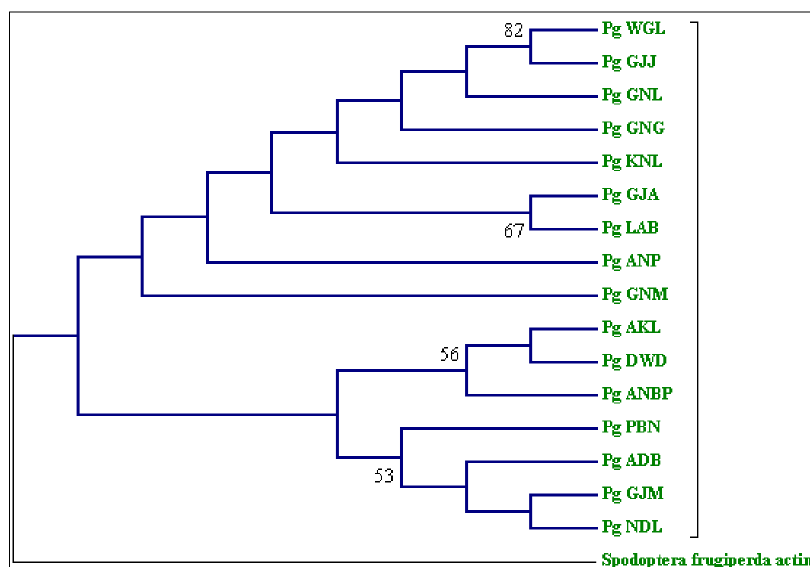


Fig 3: Neighbour joining tree constructed based on T3P parameter for 16 populations of *P. gossypiella*

βActin, genes are considered as housekeeping genes and which has to be normalized while studying expression analysis of any of the genes in plants or insects. Studies on genetic diversity and phylogenetic utility of *β actin* in insects were scarce. In present study low pair wise genetic distance (0.000 to 0.038) (Table 4) and low nucleotide diversity (0.0147) was observed when 16 populations of *P. gossypiella* were sequenced and analyzed using Tamura-3-parameers. Tajima's D value (-0.9123) is negative indicates excess of low frequency polymorphisms due to expansion of population size or due to selection even in *β Actin* gene. Phylogenetic analysis reveals that low diversity as a result all *P. gossypiella* populations grouped in to one cluster *vis a vis S. frugiperda* which is showing similarity of 91% observed among 14 populations of *P. gossypiella* when analyzed using *β tubulin* gene. Low nucleotide diversity (0.0668) of *P. gossypiella* with *β tubulin* gene. Overall results of phylogeographical analysis using housekeeping genes *β actin*, shows that very low pair-wise distance and low nucleotide diversity in *β actin.* Though, nuclear protein coding genes showed high genetic differentiation than mitochondrial gene there was not much differentiation in the values. The results in the present study revealed that mitochondrial DNA proved as promising marker for population structure and genetic diversity studies in insects. Moreover, nuclear protein coding genes can also be used as efficient molecular marker for studying population structures and demographic analysis in insects.

Phylogenetic analysis by cloning and sequence characterization in diamond back moth *(Plutella xylostella*) using actin, vATPase, tyrosine hydroxylase, ultra spiralcle protein showed that Actin genes are highly conserved across the Lepidoptera (Ashokan *et al.,* 2014). Low genetic diversity was observed among the Populations of *P. gossypiella* in India when amplified for COI gene and Haplotype H1 is recorded as ancestral one rest all are present in low frequency polymorphisms this is due to expansion of population (Sridhar *et al.,* 2016). Low pair-wise genetic distance and high genetic polymorphism were recorded in *P. gossypiella* populations when they are amplified with RAPD markers. (Shahanaz and Kalia VK, 2019) [3]. Further, in South India, 16 different

populations of *P. gossypiella* were studied for genetic diversity using COI gene and low genetic distance (0.000 to 0.058) was observed among the Populations (Chowdhary *et al*., 2017) [11]. However, the nuclear protein coding DNA in pink bollworm shows very low genetic variability when amplified using EF-1α gene (Shahanaz and Kalia VK, 2019) [3]. This study clearly shows that nuclear protein genes are highly conserved regions so they can be used to study phylogenic studies genetic differentiation among the different insect orders

Table 3: Sequences of Pink boll worm Actin gene submitted in NCBI and accession numbers

S. No	Population	Sample id	NCBI Accession Numbers
			β Actin
1	Adilabad	Pg ADB	MG182691
2	Warangal	Pg WGL	MH362665
3	Macherla	Pg GNM	MH363705
4	Lam Farm	Pg GNL	MH363706
5	Kurnool	Pg KNL	MH362669
6	Anantapur	Pg ANP	MH363703
7	Pamidi	Pg ANBP	MH362672
8	Dharwad	Pg DWD	MH362673
9	Akola	Pg AKL	MH362671
10	Parbhani	Pg PBN	MH423440
11	Anand	Pg GJA	MH362668
12	Junagarh	Pg GJJ	MH362667
13	Manglor	Pg GJM	MH362666
14	Sri Ganganagar	Pg GNG	MH363704
15	Lab (IARI)	Pg LAB	MH362674
16	Field (IARI)	Pg NDL	MH362670

Table 4: Pair-wise distance based on Tamura -3-Parameter among *P. gossypiella* populations using Actin gene.

	WGL	GJM	GJJ	GJA	KNL	NDL	AKL	ANBP	DWD	LAB	ANP	GNG	GNM	GNL	PBN	ADB
Pg WGL		0.005	0.000	0.005	0.005	0.006	0.005	0.005	0.005	0.006	0.004	0.003	0.006	0.004	0.005	0.007
Pg GJM	0.015		0.005	0.006	0.006	0.002	0.003	0.003	0.002	0.007	0.004	0.005	0.005	0.006	0.003	0.005
Pg GJJ	0.000	0.015		0.005	0.005	0.006	0.005	0.005	0.005	0.006	0.004	0.003	0.006	0.004	0.005	0.007
Pg GJA	0.013	0.020	0.013		0.006	0.007	0.006	0.006	0.006	0.006	0.005	0.005	0.006	0.006	0.006	0.008
Pg KNL	0.013	0.020	0.013	0.017		0.007	0.006	0.007	0.007	0.008	0.006	0.005	0.006	0.006	0.006	0.008
Pg NDL	0.017	0.002	0.017	0.022	0.022		0.004	0.004	0.003	0.008	0.005	0.005	0.006	0.006	0.004	0.005
Pg AKL	0.011	0.004	0.011	0.015	0.020	0.007		0.003	0.002	0.007	0.003	0.005	0.005	0.005	0.003	0.005
Pg ANBP	0.015	0.004	0.015	0.020	0.024	0.007	0.004		0.002	0.007	0.004	0.005	0.006	0.006	0.004	0.006
Pg DWD	0.013	0.002	0.013	0.017	0.022	0.004	0.002	0.002		0.007	0.004	0.005	0.005	0.005	0.004	0.005
Pg LAB	0.022	0.026	0.022	0.017	0.035	0.029	0.024	0.026	0.024		0.006	0.006	0.008	0.007	0.007	0.009
Pg ANP	0.007	0.009	0.007	0.011	0.020	0.011	0.004	0.009	0.007	0.020		0.004	0.005	0.004	0.004	0.006
Pg GNG	0.004	0.011	0.004	0.013	0.013	0.013	0.011	0.015	0.013	0.022	0.007		0.005	0.004	0.005	0.006
Pg GNM	0.017	0.013	0.017	0.017	0.020	0.015	0.011	0.015	0.013	0.031	0.011	0.015		0.006	0.005	0.007
Pg GNL	0.007	0.017	0.007	0.015	0.020	0.020	0.013	0.017	0.015	0.024	0.009	0.007	0.020		0.006	0.007
Pg PBN	0.015	0.004	0.015	0.020	0.020	0.007	0.004	0.009	0.007	0.024	0.009	0.011	0.013	0.017		0.005
Pg ADB	0.024	0.013	0.024	0.029	0.029	0.015	0.013	0.017	0.015	0.038	0.017	0.020	0.022	0.026	0.013	

References

1. Dhurua S, Gujar GT. Field evolved resistance to Bt toxin Cry1Ac in the pink bollworm, Pectinophora gossypiella (Sauders) (Lepidoptera: Gelechiidae), from India. Pest Management Science. 2011;67:898-903.
2. Naik VCB, Kumbhare S, Kranthi S, Satija U, Kranthi KR. Field evolved resistance in pink bollworm, Pectinophora gossypiella (Saunders), (Lepidoptera: Gelechiidae), to transgenic Bacillus thuringiensis (Bt)cotton expressing crystal 1Ac (Cry1Ac) and Cry2Ab in India. Pest Management Science. 2018;74(11):2544-2554.
3. Shahanaz, Kalia VK. Phylogeographical analysis of Elongation Factor 1α gene in Pectinophora gossypiella (Gelechiidae: Lepidoptera). Journal of Entomology and Zoology studies. 2019;6(5):2290-2297.
4. Gujar GT, Khawale RN, Kalia V. Genetic variability of Helicoverpa armigera (Hubner) attributable to cadherin gene-specific molecular markers. Current Science. 2007;92:800-804.
5. Thompson JD, Higgins DG, Gibson TJ. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research. 1994;22(22):4673-4680.
6. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular Biology and Evolution. 2016;33(7):1870-1874.
7. Ellango R, Asokan R, Rebijith KB, Riaz Mahmood, Ramamurthy VV. Cloning and Sequence Analyses of Four Important Genes of *Plutella xylostella* (L.) (Lepidoptera: Yponomeutidae). Entomological News. 2014;123(5):348-357. 2014.
8. Rebijith KB, Asokan R, Krishna V, Krishna Kumar NK, Ramamurthy VV. Development of species specific markers and molecular differences in mitochondrial and nuclear Dna sequences of Aphis gossypii and Myzus persicae (Hemiptera: Aphididae). Florida Entomologist. 2012;95(3):674-682.

9. Shahanaz, Kalia VK. Phylogeographical analysis of Elongation Factor 1α gene in Pectinophora gossypiella (Gelechiidae: Lepidoptera). Journal of Entomology and Zoology studies; c2019.
10. Shahanaz Kalia VK. Genetic diversity of pink bollworm, pectinophora gossypiella (Saunders) (Lepidoptera: Gelechiidae) populations in India using RAPD markers. Indian Journal of Entomology. 2019;81(2):384-389
11. Chowdhry RL, Bheemanna M, Hosmani AC, Prabhuraj A, Naik MK, Nidagundi JM. Genetic variation of mitochondrial DNA in South Indian populations of Pectinophora gossypiella (Saunders) (Lepidoptera: Gelechiidae). Journalof Entomology and Zoological studies. 2017;5(2):258-262.
12. Liu L. BEST: Bayesian estimation of species trees under the coalescent model. Bioinformatics. 2008 Nov 1;24(21):2542-3.
13. Behura SK. Molecular marker systems in insects: current trends and future avenues. Molecular ecology. 2006 Oct;15(11):3087-113.
14. Sutrisno H. Evolution of a wingless gene and its utility for inferring the relationships within Glyphodes moths. HAYATI Journal of Biosciences. 2006 Dec 1;13(4):145-50.