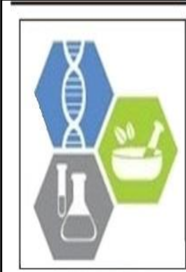

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Phylogeographical analysis of Elongation Factor 1α gene in *Pectinophora gossypiella* (Gelechiidae: Lepidoptera)

Shahanaz and Vinay K Kalia

Abstract
Pink bollworm, *Pectinophora gossypiella* is one of the most important pests of cotton. Since 2015 onwards this pest is causing havoc in the Bt cotton growing regions of India. To find out genetic variation in the seven populations of *P. gossypiella,* present study was carried out using the nuclear protein coding gene, elongation factor 1 alpha (EF-1α). Phylogeographical pattern clearly indicates that there is low genetic variation among the *P. gossypiella* populations. Nucleotide diversity and overall mean distances were 0.0063 and 0.004. Negative Tajima's D value indicates that the presence of low frequency polymorphism reflected as population expansion. Neighbor joining tree constructed based on the EF-1α sequences generated two clusters separating populations Lab, Dharwad, New Delhi in to one cluster, while Adilabad, Guntur, Gujrat-manglor, akola in to another cluster.

Keywords: *Pectinophora gossypiella*, Pink bollworm, Genetic diversity, Elongation Factor-1alpha

Introduction
The pink bollworm (PBW) *Pectinophora gossypiella* (Saunders) is native to Asia, but has become an invasive species in most of the world's cotton-growing regions [1]. At present, it has been recorded in nearly all cotton-growing countries and is a serious pest in many of these areas. With the commercialization of Bt transgenic cotton since 2002 in India and its rapid expansion throughout the country, *Helicoverpa armigera,* earlier considered to be a major pest has been relegated to secondary status whereas pink bollworm has attracted major concern being monophagous pest. The female moth lays eggs on buds, calyx and cotton bolls, on hatching larvae enter the bolls. They wad through the cotton lint to feed on the seeds. Since cotton is used for both fiber and seed oil, the damage is twofold. Steady increase in its incidence in the later stage of the crop reduces the cotton yield to the extent of 4 to 5 q/ha leading to low quality lint.

In parts of India, PBW has developed resistance to Bt transgenic cotton (Bollgard-I) expressing Cry1Ac toxin in some localities of Gujarat just after eight years of commercialization of Bt cotton in India [2, 3]. Even second generation Bt cotton (Bollgard-II) which contains two toxins (Cry1Ac + Cry2Ab) is severely damaged by pink bollworm and yield-losses were observed since 2015 onwards in Gujarat, Andhra Pradesh, Telangana and Maharashtra [4]. Increased frequency of PBW resistance to Bt cotton is reported in China [5].

Substantial work has been reported on resistance monitoring in *H. armigera* being its importance as polyphagous pest [6, 7, 8, 9] but for PBW so far little information is available in India. Despite the serious damage caused by PBW around the world, few studies have assessed genetic diversity using mitochondrial cytochrome C oxidase (COX I) gene [10] and population structure using microsatellite markers [11] which is essential to understand the pest for developing management strategies.

It is commonly assumed that mitochondrial DNA (mtDNA) evolves at a faster rate than nuclear DNA (nu DNA) and their inheritance pattern found to be different [12]. Nuclear protein coding genes may be promising candidates, since they are more conserved, evolve at a slower rate than mitochondrial coding genes, show little length variability, easily align able and exhibit variable rates of substitution both within and among genes [13, 14]. However, the problem with nuclear gene is that they occur in lower numbers. Recently, a few protein coding genes have become of wider use, among these Elongation factor-1a (EF-1 α) has proved useful to infer the relationship from species to subfamily level within lepidopteran [15].

EF-1α is a protein involved in the GTP dependent binding of charged transfer (+) RNAs to the acceptor site of the ribosome during protein synthesis. After the binding of the complex, the GTP ase activity of EF-1α is activated by the ribosome and GTP hydrolysis leads to the dissociation of EF-1α from the tRNA, permitting the full entrance of the t RNA into the A site [16, 17]. EF-1α was originally identified as a single- copy gene in insects. Later it was found that stage and tissue-specific isoforms are present in some insect orders *viz.,* Coleoptera, Hymenoptera, Diptera, Thysanoptera, Hemiptera - Coccoidae and members of the Neuropterida [18]. In Contrast to the above Hemiptera [19], Lepidoptera [20] and Odonata [21] have a single copy of gene. “Say Goodbye to Tribes in the New House Fly Classification” call given by [22]. They scrutinized four genes [mitochondrial COI; nuclear AATS, CAD (region 4) and *EF-1α*] from 142 species in 67 genera representing all tribes across the biogeographic regions in Muscidae and revised classification with only three subfamilies without tribes in Muscidae. Genetic variability of *Deanolis sublimbalis* belonging to different locations of Andhra Pradesh using EF-1 α and reported no genetic variation among the populations estimated by [23]. However, EF-1α has been identified as a potentially useful gene for studies of higher level Populations phylogenetic relationships and genetic variability studies especially in insects due to the conserved nature of the amino acid sequence among these organisms.

Based on this background knowledge, we used nu DNA that have been widely used in many insect population genetic studies to detect the genetic diversity, genetic differentiation and population structure of *P. gossypiella* from southern cotton growing zones of India and compared with two populations collected from northern zone.

Materials and Methods

Sample Collection

Full grown larvae of *P. gossypiella* were separated from infested bolls of cotton belonging to seven different locations of India during 2016-17 (Table 1). Five individuals from each location were collected, starved overnight, surface sterilized with 70% ethanol and transferred to the microcentrifuge tube (MCT) and stored at – 80 ^{0}C to carry out the molecular studies.

Table 1: Details of *Pectinophora gossypiella* sampled for genetic variability studies during 2016

S. No	Zone	Location		Date of Sampling	Geographical Coordinates	Population Code
		State	Place			
1	South India	Telangana	Adilabad	24-Nov-2016	19.6808 N 78.5359 E	ADB
2		Andhra Pradesh	Macherla	28-Nov-2016	16.4773 N 79.4375 E	GNM
3		Karnataka	Dharwad	23-Dec-2016	15.4461 N 74.9986 E	DWD
4	Central India	Maharashtra	Akola	12-Jan-2016	20.6962 N 77.0589 E	AKL
5		Gujarat	Manglor	18-Dec-2016	21.1329 N 70.1154 E	GJM
6	North India	Rajasthan	Sri Ganganagar	11-Nov-2016	29.9038 N 73.8771 E	GNG
7		Delhi	Lab (IARI)	30-Dec-2016	28.6448 N 77.2167 E	LAB

DNA extraction, amplification and sequencing

Genomic DNA was extracted from the single healthy individual larvae from each location following the method described by [7] with minor modifications. Individual larvae was crushed with a sterilized pestle to a fine powder immediately after removing from – 80 ^{0}C to avoid thawing. Then 400 µl pre-warmed (at 60 ºC for 10 minutes) DNA extraction buffer (2% Cetyl trimethyl ammonium bromide, 1.4 M NaCl, 100 mM Tris HCl, 20mM EDTA) and 4 µl β-mercapto ethanol was added to the homogenized larvae. Samples were incubated in a dry bath at 65 ºC for 1 hr with gentle shaking at ten minute interval. After incubation, an equal volume of chloroform: isoamyl alcohol (24: 1v/v) was added and centrifuged at 12000 rpm for 10 minutes at room temperature after proper mixing by gentle inversion. Then aqueous phase was transferred to fresh MCT, to which 0.6 volumes of Isopropanol and 150µl of 4M NaCl were added and the contents were mixed by gentle inversion. The Samples were incubated for an hour at room temperature for DNA precipitation followed by centrifugation at 10000 rpm for 10 minutes. Supernatant was decanted and the pellet was washed twice with 70% ethanol. DNA pellet was air dried and dissolved in 50µl of nuclease free water. DNA samples were incubated at 37 ºC for 1 h with the addition of RNAase to the samples. The DNA obtained was checked by running on 0.8% agarose gel. The DNA samples were stored at -20º C till further use.

The extracted DNA was subjected to PCR amplification of 550 bp region by using EF-1α primers (Forward primer: 5’-GCCAACATCACCACTGAA-3’ and Reverse primer: 5’-CTAGCTACTTCTTGCCCTTG-3’) in GenPro (Biover) Thermal Cycler. The 50 µl amplification reaction mixture consists of 2 µl of 10 pmol of each primer, 5.0 µl of 1X PCR Buffer (10 mM Tris/HCl, 50 mM KCl, 2 mM Mgcl2), 4 µl of 2.5 mM of each dNTP and 1U of Taq polymerase including 2 µl template DNA. PCR was performed with an initial denaturation at 94 ºC for 5 min followed by 40 cycles of denaturation for 1min at 94 ºC, annealing for 1 min at 58 ºC, extension at 72 ºC for 2 min and a final extension of 10 min at 72 ºC. The PCR amplified products were resolved on 1.2% agarose gel stained with ethidium bromide and visualized in gel documentation system. The amplified PCR products were sequenced (Chromus Biotech Pvt. Ltd, Bengaluru, India).

Phylogenetic Analysis

The EF-1α sequences obtained from the both stands in FASTA format were aligned using ClustalW programme 24 implemented in MEGA 7.0 software package sequence alignment application [25]. The raw sequences were trimmed, aligned and used for diversity studies. Multiple sequence alignments were performed with ClustalW algorithm using default parameters. The EF-1α sequences were subjected to homology search using Basic Local Alignment Search tool of the National Centre for Biotechnology Information (NCBI) using a non redundant nucleotide database and protein database in balstn and blastx searches. A Neighbor joining (NJ) phylogenetic tree was constructed based on the BLAST similarity search results taking GenBank database of 10 different species belongs to Lepidoptera**.** Alignment gaps within aligned regions were treated as missing data. The

phylogenetic relationship among the populations was inferred with MEGA 7.0 using neighbor joining (NJ) method under a distance model of Kimura 2 parameter. Robustness of the tree was evaluated by bootstrapping with 1000 replicates.

Results and discussions

A sequence length of 550 bp was obtained when both forward and reverse EF-1α primers were used. Sequences of seven populations were aligned in CLUSTAL W after removing gaps, a 505 bp region used for analysis purpose (Fig 1). The nucleotide sequence of the EF-1α gene was used in a similarity search performed with the Blastn programme. Similarity search result indicates that *P. gossypiella* has a maximum identity with most of the insect species of Lepidoptera. Phylogenetic tree constructed based the sequence of *P. gossypiella* EF-1α shows high identity with the EF-1α mRNA sequences of different insect species belongs to Lepidoptera (upto 89-94 % with E value 0.0). Furthermore, the nucleotide sequence presents 96% identity with the *Pexicopia malvella*, the only Gelechiidae EF-1α mRNA sequence currently available in the nucleotide database of the NCBI. Whereas, EF-1α gene sequence is showing just 89% identity with the Butterflies, *Papilio polytes* and *P. xuthus*. Substantial resolution of recovered branches at genus and species level in Lepidoptera obtained through the EF-1α gene [20, 26]. In the present study also EF-1α recovered *P. gossypiella* populations till genus level.

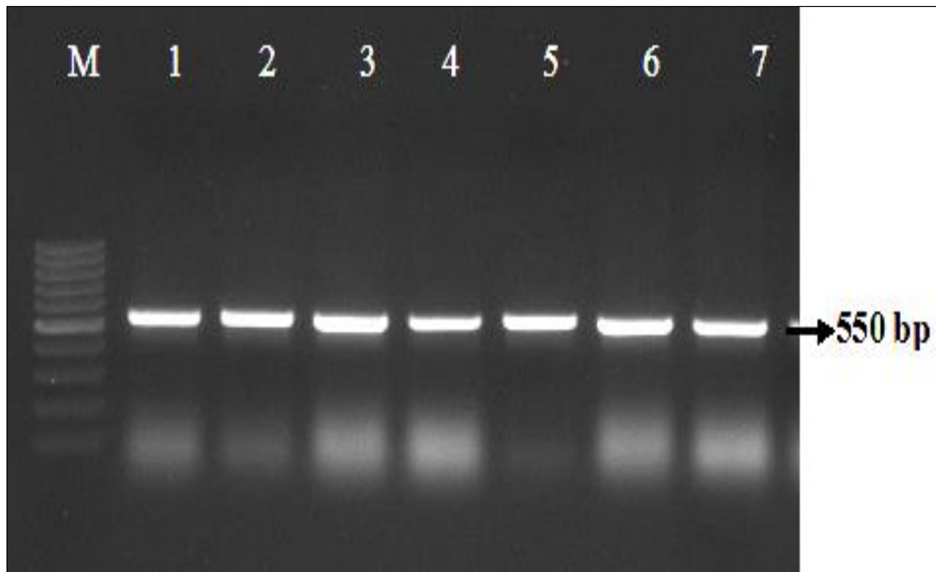

Fig 1: PCR amplified product of EF-1α gene of *Pectinophora gossypiella* resolved on 1.2 % agarose gel from seven locations of India. M: Marker, Lane 1: Adilabad, 2: Guntur, 3: Manglor, 4: Lab, 5: Dharwad, 6: Sri Ganganagar, 7: Akola

Neighbor joining tree constructed with seven populations of *P. gossypiella* and ten different insect species from GenBank database reveals that total seven clades were formed. Total branch length of phylogenetic tree is 0.295 base substitutions per site. Major clade formed clearly differentiates the EF-lα gene of moths from butterflies (Fig 2). The 2nd and 3rd clades differentiate the insect species according to families. 3rd, 4th and 5th subclades of clade 2 grouped the insects of Gelechiidae upto tribe level.

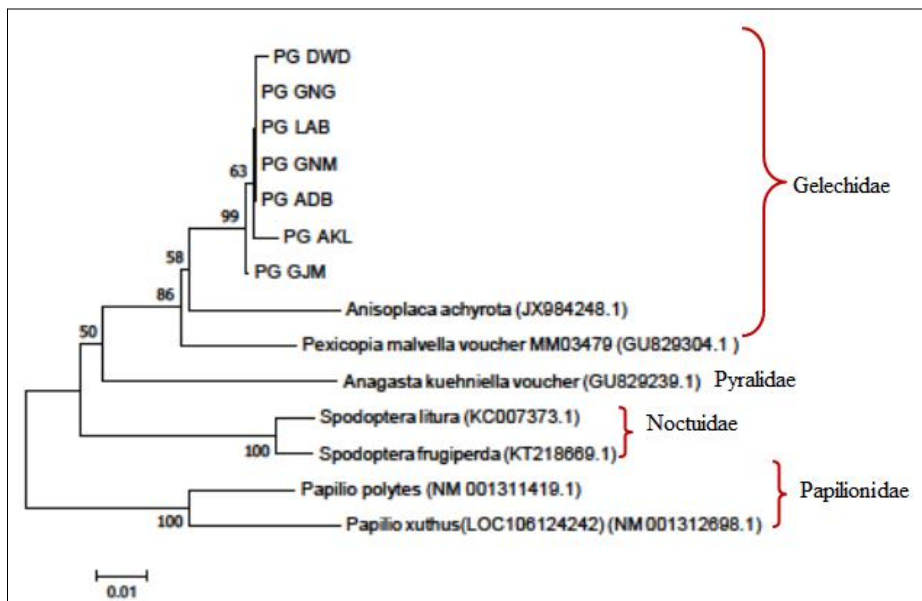

Fig 2: Neighbor joining tree constructed based on EF-1α gene sequences of different populations of *Pectinophora gossypiella* taking different lepidopteran insects as outliers

To validate the best phylogenetic tree, Maximum likelihood tree was also constructed which showed one major clade separating moths and butterflies like NJ tree (Fig. 3). The first subclade of major clade have two sub clades which are arranged as per the family.

There is not much difference between the phylogenetic tree constructed which are based on the Neighbor joining and Maximum likelihood parameters.

Further, if we exclude the seven outliers groups, then phylogenetic tree recognizes only one major clade among seven populations of *P. gossypiella* containing two sub clades. Similar results were obtained in the case of *D. sublimbalis* where phylogenetic tree constructed based on Neiborjoining method separated all populations from outer group *Maruca vitrata* [23].

Subtree of Neighborjoining tree reveals that *P. gossypiella* populations from Adilabad, Guntur, Akola, Manglor were grouped under first subclade with branch length of 0.001 base substitutions per site (Fig. 4). Whereas, second subclade consists of populations from lab (IARI), Dharwad, Sri Ganganagar with same branch length like the first sub clade.

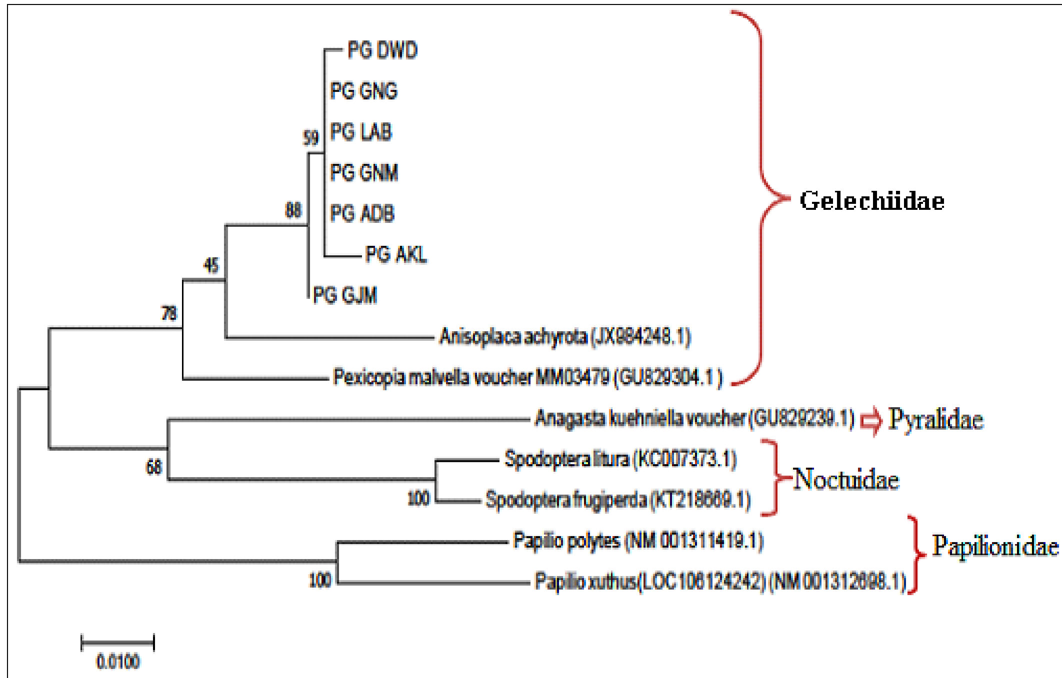

Fig 3: Maximum Likelihood tree constructed based on EF-1α gene sequences of different populations of *Pectinophora gossypiella* taking different lepidopteran insects as outlier

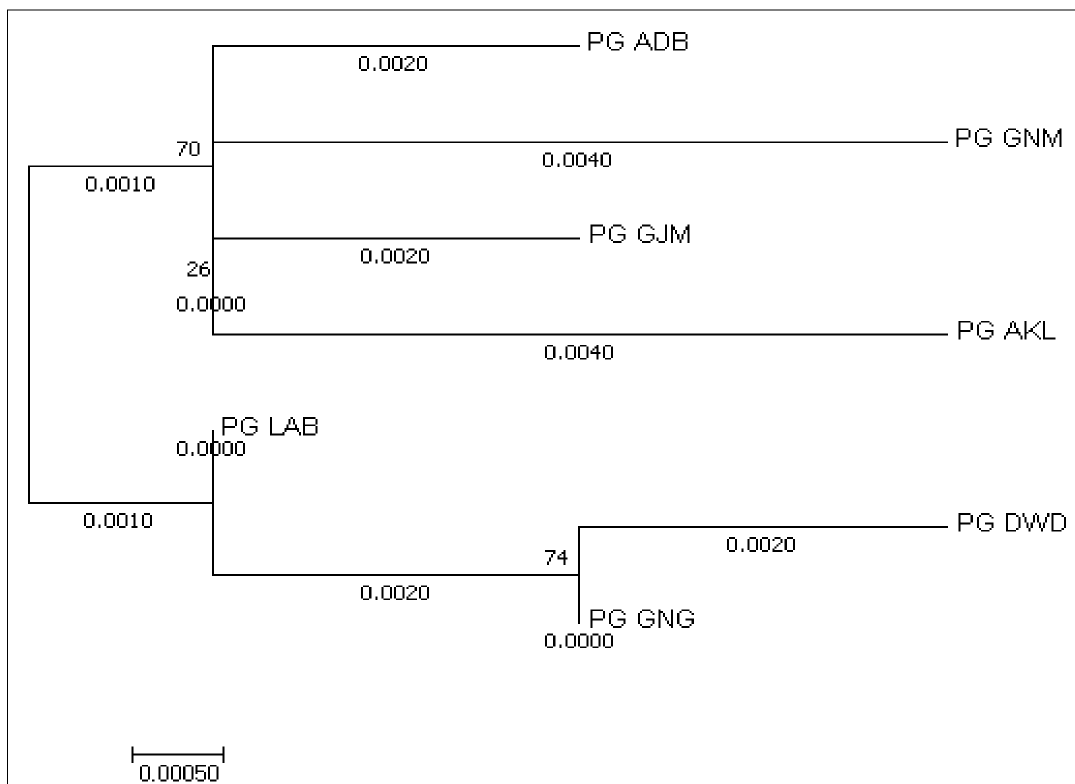

Fig 4: Sub-tree Neighbor-joining tree of EF-1α sequences of *Pectinophora gossypiella* populations based on Kimura2 parameter distances.

Divergence among the individuals were calculated using Kimura 2 parameter are in the units of the number of base substitutions per site and depicted graphically through a neighbor joining tree constructed using MEGA 7.0. Overall mean distance of seven populations was 0.004 ± 0.001. The nucleotide composition analysis showed that A=23%, T=19.75%, C=31.2%, G=26% in, sequence region showed high GC rich nucleotide composition. The estimated transition to transversion ratio was (R=0.315). In contrast to that molecular study of 20 populations of lac insect using combined sequences of mitochondrial cytochrome C oxidase sub unit I, small subunit ribosomal RNA gene loci, Elongation factor 1 alpha reveals that tamura- Nei distance 9.8 % within group taxa and an excess of AT rich (62%) and transition and trans version bias was 1.05 [27].

Further demographic values like nucleotide diversity (Pi), 0.00603 and Tajima's D statistic value is calculated from the number of segregating sites (S=9) is -0.9054. A non-significantly negative value indicates that an excess of low frequency polymorphisms indicating population size expansion or selection. Genetic distance among the populations of *P. gossypiella* is calculated using pairwise Kimura 2 parameter gave values ranging from 0.002 to 0.01. Highest genetic distance was observed between Guntur, Akola *vis a vis* Dharwad, whereas lowest genetic distance was observed between Dharwad *vis a vis* Sri Ganganagar and Lab. Consequently, relatively low genetic diversity and weak genetic structure was observed in case of *Coenosia attenuate* a predator fly sampled from 52 localities belongs to 17 countries [28] when amplified using EF-1α and mitochondrial COI gene. Similarly, studies on molecular diversity of populations of aphids *viz., Myzus persicae*, and *Aphis gossypii* belongs to different host plants and geographical locations of India using COI, COII and EF-1α reveals very few nucleotide variations among them and no cryptic species nor biotypes existing in India [29]. However, *D. sublimbalis* populations belonging to different regions of Andhra Pradesh did not show any variation among the sequences, nucleotide diversity and overall mean distances were zero and transition and transversion bias found to be 0.49 [23]. Studies on genetic variation *of M. persicae* complex in three continents using CoII and EF-1α reveals that population within the *M. persicae* complex were identical [30].

Table 2: Pairwise genetic distance among the Populations of *Pectinophora gossypiella*

Population	ADB	GNM	GJM	LAB	DWD	AKL	GNG
ADB	-						
GNM	0.006	-					
GJM	0.004	0.006	-				
LAB	0.004	0.006	0.004	-			
DWD	0.008	0.010	0.008	0.004	-		
AKL	0.006	0.008	0.006	0.006	0.010	-	
GNG	0.006	0.008	0.006	0.002	0.002	0.008	-

Multiple alignments of the 177 amino acid deduced sequence with EF-1α amino acid deduced sequences of other insect species (Table 3) revealed significant similarity (96-98% identity) with the sequences of *P. polytes* (EF-1α*), P. xuthus*, *A. chryota P. malvella*, (EF-1α), *A. kuehniella* (EF-1α) and *S. litura* (EF-1α), respectively.

The sequences obtained from the present study were deposited in Gen Bank (Accession numbers: MH329234-38, MH329241, MH329244). *EF-1α* proteins consist of three structural domains: domain I or G domain, domain II, and domain III. Domain I (G domain) is approximately 200 residues long, is responsible for binding either GTP or GDP and is related to the GTPase domains of other G proteins. Domains II and III both contain approximately 100 residues and are β barrels These domains are in the same relative orientation and might act as a single rigid unit during hydrolysis of GTP. *EF-1α* domains III is present in the deduced amino acid sequence of *P. gossypiella.* The structures show that *EF-1α* undergoes a large conformational change upon hydrolysis of GTP to GDP, particularly in the switch I and switch II regions of the G domain, causing a substantial rotation of the G domain with respect to domains II and III. A highly conserved structure of *EF-1α* reported between eukaryotes and arachea.

Populations of *P. gossypiella* showed low genetic diversity in

Table 3: Deduced amino acid sequence of EF-1α of *P. gossypiella* and other related species *S. litura, P. malvella, A. chyrota, P. polytes, P. xuthus, A. kuhneilla* .Letters highlighted in yellow are polymorphi cites

	*	*	*	*	*	*	*	*	*	*	*	*		*	*	*	*	*		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
P. gossypiella	G	Y	V	A	G	D	S	K	N	N	P	P	K	G	A	A	D	F	T	A	Q	V	I	V	L	N	H	P	G	Q	I	S	N	G	Y	T	P	V	L	D
S. litura	G	Y	V	A	G	D	S	K	N	N	P	P	K	G	A	A	D	F	T	A	Q	V	I	V	L	N	H	P	G	Q	I	S	N	G	Y	T	P	V	L	D
P. malvella	G	Y	V	A	G	D	S	K	N	N	P	P	K	G	A	A	D	F	?	A	Q	V	I	V	L	N	H	P	G	Q	I	S	N	G	Y	T	P	V	L	D
A. achyrota	G	Y	V	A	G	D	S	K	N	N	P	P	R	G	A	A	D	F	N	A	Q	V	I	V	L	N	H	P	G	Q	I	S	N	G	Y	T	P	V	L	D
P. polytes	G	Y	V	A	G	D	S	K	N	N	P	P	K	G	A	A	D	F	T	A	Q	V	I	V	L	N	H	P	G	Q	I	S	N	G	Y	T	P	V	L	D
P. xuthus	G	Y	V	A	G	D	S	K	N	N	P	P	K	G	A	A	D	F	T	A	Q	V	I	V	L	N	H	P	G	Q	I	S	N	G	Y	T	P	V	L	D
A. kuehniella	G	Y	V	A	G	D	S	K	N	N	P	P	K	G	A	A	D	F	T	A	Q	V	I	V	L	N	H	P	G	Q	I	S	N	G	Y	T	P	V	L	D
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P. gossypiella	C	H	T	A	H	I	A	C	K	F	A	E	I	K	E	K	V	D	R	R	T	G	K	S	T	E	D	N	P	K	S	I	K	S	G	D	A	A	I	V
S. litura	C	H	T	A	H	I	A	C	K	F	A	E	I	K	E	K	V	D	R	R	T	G	K	S	T	E	D	N	P	K	S	I	K	S	G	D	A	A	I	V
P. malvella	C	H	T	A	H	I	A	C	K	F	A	E	I	K	E	K	V	D	R	R	T	G	K	S	T	E	D	N	P	K	S	I	K	S	G	D	A	A	I	V
A. achyrota	C	H	T	A	H	I	A	C	K	F	A	E	I	K	E	K	V	D	R	R	T	G	K	?	T	E	D	N	P	K	S	I	K	S	G	D	A	A	I	V
P. polytes	C	H	T	A	H	I	A	C	K	F	A	E	I	K	E	K	V	D	R	R	T	G	K	S	T	E	D	N	P	K	S	I	K	S	G	D	A	A	I	V
P. xuthus	C	H	T	A	H	I	A	C	K	F	A	E	I	K	E	K	V	D	R	R	T	G	K	S	T	E	D	N	P	K	S	I	K	S	G	D	A	A	I	V
A. kuehniella	C	H	T	A	H	I	A	C	K	F	A	E	I	K	E	K	V	D	R	R	T	G	K	S	T	E	D	N	P	K	S	I	K	S	G	D	A	A	I	V
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P. gossypiella	N	L	V	P	S	K	P	L	C	V	E	S	F	Q	E	F	P	P	L	G	R	F	A	V	R	D	M	R	Q	T	V	A	V	G	V	I	K	S	V	H
S. litura	N	L	V	P	S	K	P	L	C	V	E	S	F	Q	E	F	P	P	L	G	R	F	A	V	R	D	M	R	Q	T	V	A	V	G	V	I	K	A	V	N
P. malvella	N	L	V	P	S	K	P	L	C	V	E	S	F	Q	E	F	P	P	L	G	R	F	A	V	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. achyrota	N	L	V	P	S	K	P	L	C	V	E	S	F	Q	E	F	P	P	L	G	R	F	A	V	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. polytes	N	L	V	P	S	K	P	L	C	V	E	S	F	Q	E	F	P	P	L	G	R	F	A	V	R	D	M	R	Q	T	V	A	V	G	V	I	K	A	V	N
P. xuthus	N	L	V	P	S	K	P	L	C	V	E	S	F	Q	E	F	P	P	L	G	R	F	A	V	R	D	M	R	Q	T	V	A	V	G	V	I	K	S	V	N
A. kuehniella	N	L	V	P	S	K	P	L	C	V	E	S	F	Q	E	F	P	P	L	G	R	F	A	V	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	*	*	*			*	*	*	*	*	*	*	*		*	*	*	*																						
P. gossypiella	F	K	E	G	A	G	G	K	V	T	K	A	A	Q	K	A	S	*																						
S. litura	F	K	E	A	G	G	G	K	V	T	K	A	A	E	K	-	-	-																						
P. malvella	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-																						
A. achyrota	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-																						
P. polytes	F	K	E	G	A	G	G	K	V	T	K	A	A	E	K	A	S	-																						
P. xuthus	F	K	E	G	A	G	G	K	V	T	K	A	A	E	K	A	-	-																						
A. kuehniella	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-																						

India when amplified using COI gene. H1 considered as the ancestral haplotype rest are low frequency polymorphisms formed due to population expansion [10]. RAPD analysis of 12 populations of *P. gossypiella* in India revealed that OPI-11 to OPI-14 primers were produced scorable amplicons. High genetic polymorphism, low pair-wise genetic distance observed among the populations of *P. gossypiella* (Shahanaz and Kalia 2018 unpublished). Further, the very low genetic variation observed in 16 different populations of *P. gossypiella* in South India using the COI gene. Low genetic distance (0.000 to 0.058) was observed among Populations [31]. However, the nuclear DNA in this species shows also very low variability in EF-1a genes. This suggests recent divergence within and population of *P. gossypiella* is unstructured in India due to recent colonization or high levels of gene flow which led to lack of Phylogeographical structure. Though the EF-1α used for phylogenetic reconstructioning of the species, it can be used as a promising molecular marker for the genetic diversity studies in insect populations.

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