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RESEARCH ARTICLE

Mitochondria COI-based genetic diversity of the cotton leafhopper *Amrasca biguttula biguttula* (Ishida) populations from India

Sandhya Kranthi[a], Amol Bharat Ghodke[a], Raghavendra K. Puttuswamy[a], Madhumita Mandle[a], Ritu Nandanwar[a], Usha Satija[a], Rishi Kumar Pareek[b], Himanshu Desai[c], Shashikant S. Udikeri[d], Dhara Jothi Balakrishna[e], Bheemanna M. Hugar[f], Dilip Monga[b] and Keshav R. Kranthi[a]

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ABSTRACT

Amrasca biguttula biguttula (Ishida), the cotton leafhopper, is a polyphagous insect pest of Asia and Southeast Asian countries. We sequenced a mitochondrial COI gene fragment from 67 individuals of cotton leafhopper collected from 7 major cotton growing states of North, Central, and South India. Genetic divergence analysis of leaf hopper population across India confirmed the presence of single species. Thirty haplotypes, in total, were determined across different regions of India. While population from North India was dominated by single haplotype, the south and central Indian populations show dispersion of different haplotypes across the region. The neutrality test rejection for the north Indian population also suggests population expansion. The genetic differentiation and gene flow analysis together confirmed the phylogeographic structure of the *A. biguttula biguttula* Ishida as isolated by distance.

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KEYWORDS
Genetic diversity; cytochrome oxidase; cotton leafhopper

Introduction

The cotton leafhopper, *A. biguttula biguttula* (Ishida) (Homoptera: Cicadellidae) is a sap-feeding insect pest of cotton in India, Pakistan, Bangladesh, China, and North Africa. Cultivation of leafhopper susceptible hybrids and reduction in conventional insecticide applications on Bt cotton, in addition to insecticide resistance were assumed to be reasons for higher incidence of leafhoppers on cotton (Kranthi et al. 2009; Prabhakar et al. 2011). Both adults and nymphs suck the sap from leaves and inject toxic saliva resulting in 'hopper burn' symptoms, which ultimately result in the loss of plant vigour and significant yield losses. *A. biguttula biguttula* found on cotton was known to be a pest of vegetative stage but in recent years it has been occurring throughout the crop growth phase, causing significant yield losses. The occurrence of leaf hoppers on Bt cotton (Raja et al. 2007; Kalkal et al. 2009; Murugesan & Kavitha 2010) and yield reduction due to this pest alone has been reported up to 50% (Atakan 2009). Substantial misuse of insecticides against the cotton leaf hopper resulted in the development of resistance in leafhoppers against organophosphates (Rajwinder & Kang 2015) and neonicotinoids (Shreevani et al. 2013).

Cicadellids are one of the most diverse families of terrestrial organisms, comprising over 25,000 described species of sap sucking insects (Dietrich et al. 1997). Among several species of leafhoppers, the cotton leafhopper *A. biguttula biguttula* (Ishida), (Hemiptera: Cicadellidae) is the dominant species on cotton in India. Populations of cotton leafhopper, *A. biguttula biguttula* (Ishida) from different geographical locations of India were found to be morphologically similar. Molecular phylogenetic analysis to ascertain the genetic difference in leafhopper populations from major cotton-growing regions of the India will pave the way and assist in designing optimal control strategies. Mitochondrial DNA (mtDNA) markers are powerful tools to assess gene flow and genetic differentiation between the species of a given population (Folmer et al. 1994). It is widely used to reveal the phylogenetic relationships of insects, particularly when morphological differentiation of populations is difficult (Simon et al. 1994). Analysis carried by researchers on animal groups showed mitochondrial DNA (mtDNA) as a most conserved region (Lunt et al. 1996) and it carries the footprints of the evolutionary relationships of insects (Simon et al. 1994). Mitochondrial cytochrome oxidase I (COI) (Hebert et al. 2003; Smith 2005) gene has been used successfully for phylogenetic analysis in leafhoppers belonging to genus *Flexamia* (Dietrich et al. 1997) *Empoasca*, *Jacobiasca* (Fu et al. 2014), and *Dalbulus* (Palomera et al. 2012).

Documentation of the information on the nature and extent of genetic variation, gene flow and genetic differentiation of populations of cotton leaf hopper depicted by molecular tools is scanty (Sagar et al. 2014). Hence, the study was carried out using mitochondrial DNA cytochrome oxidase

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I (COI) region to elucidate the genetic diversity and phylogeographic structure of leafhopper populations from seven major cotton growing states covering eighteen major cotton growing districts of India. The current study aimed to determine the intraspecific genetic diversity of *A. biguttula biguttula* and evaluate the extent and nature of genetic variation in leaf hopper populations in India. Sixty-seven samples of *A. biguttula biguttula* (Ishida) were collected on cotton. Sequences of mtCOI gene were amplified, sequenced, analysed, and the genetic variation was elucidated in the selected populations.

Material and methods

Cotton leafhopper collection

Leafhopper nymphs (largely 2nd instar) were sampled on cotton, from seven major cotton growing states of North, Central, and South India covering 18 major cotton growing districts (Table 1 and Figure 1). All samples were collected in 95% ethanol and stored at 4 °C.

DNA isolation

Single insect was crushed in 50 µl of ice cold homogenization buffer. Lysis buffer (20ul) was added and kept at room temperature for 5–10 min. Samples were incubated at 65 °C for 30 min. RNase (5ul) was added and again incubated for 20 min. at 37 °C. Potassium acetate (20ul) was added and incubated at 4 °C for 30 min. All the samples were centrifuged and 100 µl of phenol: chloroform: isoamyl alcohol (25:24:1) mixture was added to the supernatant and then again centrifuged. The aqueous phase was removed and added to 100 µl of ice cold isopropanol; and incubated overnight at −20 °C. Samples were centrifuged and the pellet was washed with 70% ethanol. Supernatant was discarded after centrifugation, pellet was air dried and dissolved in 50 µl of TE buffer. All the centrifugation steps were carried out at 12,000 rpm at 4 °C for 10 min.

Amplification of cytochrome oxidase I (COI) gene and sequencing

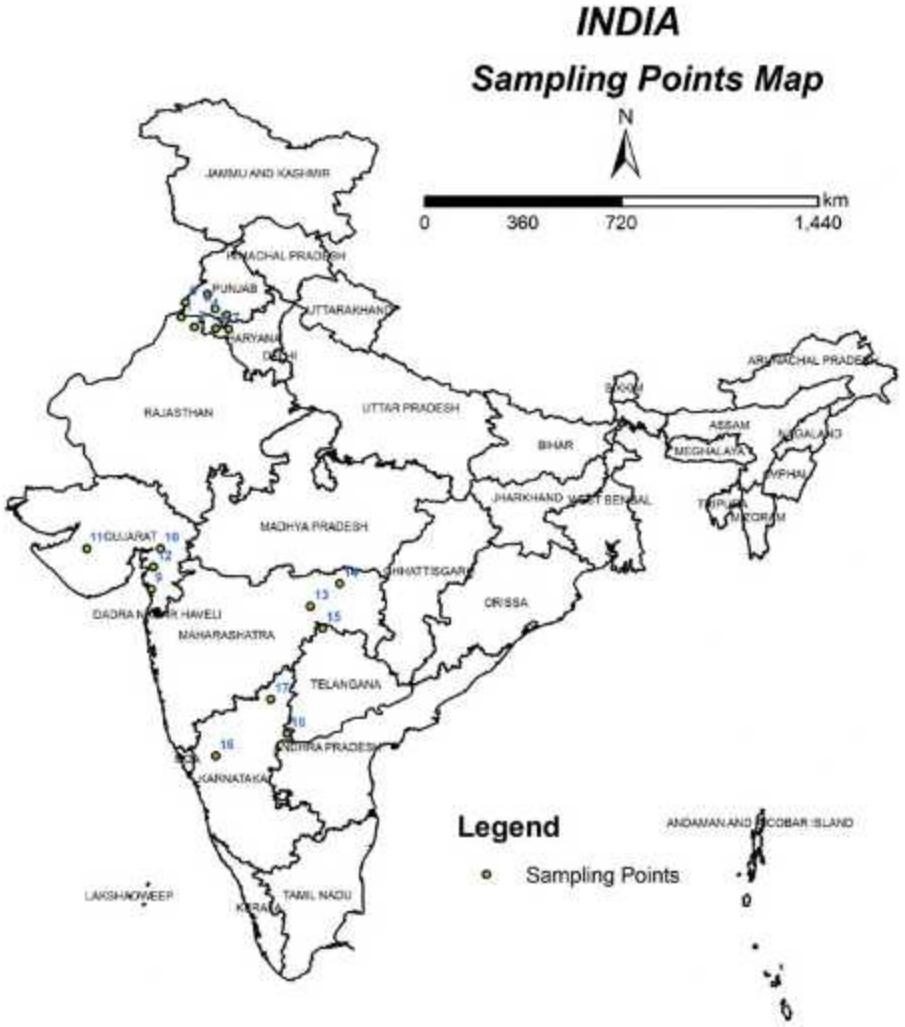
The primers for amplification of partial COI region were designed (Folmer et al. 1994): forward primer: 5′-GCT CAA CAA ATC ATA AAG ATA TTG G-3′ and reverse primer: 5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3. PCR amplifications were performed in a 25 µl reaction mixture containing 1 µl of DNA sample, 1X PCR buffer (10 mM Tris-HCl buffer, 50 mM KCl and 2% gelatin), 2 mM $MgCl_2$, 0.1 mM of each dNTP and 10 pmoles of each primer and 1U of Taq polymerase (Genei™). The PCR amplification programme was as follows: 94 °C for 2 min, 94 °C for 45 s, 50.8 °C for 45 s, 72 °C for 1.5 min (38 cycles) with final extension at 72 °C for 10 min. PCR-amplified products were subjected to 1.2% agarose gel electrophoresis and stained in ethidium bromide. The expected size of the bands on the agarose gel were excised and purified with QIAquick Gel Extraction kit (Qiagen®). Gel-purified samples were sequenced (both strands) through custom-sequencing service of M/S Merck Specialties Private Limited (Merck Genei™) (Bengaluru India) using COI forward and reverse primer on automated ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) Bio edit v7.1.9. The sequences were edited using Bio edit.

Genetic divergence, genetic differentiation, gene flow studies, and neutrality tests

Haplotype diversity (Hd), nucleotide diversity (pi), polymorphic sites, number of haplotypes, haplotype group,

Table 1. Collection sites of leaf hopper nymphal populations.

State	Sample code	District	Latitude (degrees minutes seconds)	Longitude (degrees minutes seconds)	Haplotype Accession number
North India					
Rajasthan (RJ)	SG1,3,6,7,11,15, 17, 20, 26,28,30,32,33,SGd11, SGd12, SGd13	Shriganganagar (SG)	29° 55′ 0″ N	73° 53′ 0″ E	KX813747–KX813759, KX813777–KX813779
	RJ-HG12	Hanumangarh (HG)	29° 35′ 0 ″ N	74° 19′ 0″ E	KX813776
Punjab (PB)	PB-BT38,BT46,BT48,BT49,BT50,BT52, BT54,BT67,BT76,BT78,BT80,BT84, BT86	Bhatinda (BT)	30° 11′ N	75° 00′ E	KX813760–KX813772
	FK107	Faridkot (FK)	30° 40′ 0″ N	74° 45′ 0″ E	KX813773
	MS12	Mansa (MS)	29° 59′ 0″ N	75° 23′ 0″ E	KX813782
	FZ13,FZ14	Fazilka district (FZ)	30° 24′ 0″ N	74° 2′ 0″ E	KX813780, KX813781
Haryana (HR)	FB12,FB14	Fatehabad (FB)	29° 31′ 0″ N	75° 27′ 0″ E	KX813774–KX813775
	SS11,SS12,SS13	Sirsa (SS)	29° 32′ 0″ N	75° 1′ 0″ E	KX813783–KX813785
Central India					
Gujarat (GJ)	SUD	Surat (SU)	20° 58′ 0″ N	72° 54′ 0″ E	KX813742
	VDJ,VDL	Vadodara (VD)	22° 18′ 0″ N	73° 12′ 0″ E	KX813743, KX813744
	RKO	Rajkot (RK)	22° 18′ 0″ N	70° 47′ 0″ E	KX813745
	BHT	Bharuch (BH)	21° 42′ 0″ N	72° 58′ 0″ E	KX813746
Maharashtra (MH)	YM7, YM9	Yavatmal (YM)	20° 24′ 0″ N	78° 8′ 0″ E	KX813740–KX813741
	NG4, NG5	Nagpur (NG)	21° 9′ 0″ N	79° 6′ 0″ E	KX813738–KX813739
South India					
Telangana (TA)	AB4, AB5, AB6, AB7, AB10 AB1, B5, B6, B9, B10 C1,C2, C3, C4, C5	Adilabad (AB)	19° 40′ 0″ N	78° 32′ 0″ E	KX813719–KX813733
Karnataka (KA)	DW12	Dharwad (DW)	15° 28′ 0″ N	75° 1′ 0″ E	KX813734
	GB2	Gulbarga (GB)	17° 20′ 0″ N	76° 50′ 0″ E	KX813735
	RC31,RC32	Raichur (RC)	16° 12′ 0″ N	77° 22′ 0″ E	KX813736–KX813737



District Name	Legend
Sriganganagar (SG)	1
Hanumangarh(HG)	2
Bhatinda (BH)	3
Faridkot (FT)	4
Mansa (MS)	5
Fazilka (FZ)	6
Fatehabad (FB)	7
Sirsa (SS)	8
Surat (SU)	9
Vadodara (VD)	10
Rajkot (RK)	11
Bharuch(BH)	12
Yavatmal (YT)	13
Nagpur (NG)	14
Adilabad (AB)	15
Dharwad (DW)	16
Gulbarga (GB)	17
Raichur (RC)	18

Figure 1. Locations of insect collection.

genetic differentiation (F_{ST}), and gene flow (Nm) values were obtained from the tests performed using DnaSP 5.1 software (Librado & Rozas 2009). Tajima's *D* (Tajima 1989), Fu and Li's *D** and *F**, and Tajima's *D* tests of neutrality were also performed using DnaSP 5.1 software to detect the range of population expansions. The levels of genetic differentiation can be categorized as F_{ST} 0.25 (great differentiation), 0.15–0.25 (moderate differentiation), and F_{ST}, 0.05 (negligible differentiation) (Wright 1978). The levels of gene flow can be categorized as Nm 1 (high gene flow), 0.25–0.99 (intermediate gene flow), and Nm 0.25 (low gene flow) (Govindaraju 1989). AMOVA and pairwise F_{ST} values for the mtDNA data set was calculated using Arlequin version 3.0 (Excoffier et al. 1992, 2005). Genetic distance (F_{ST}) values from the above and geographic distance representing selected population groups such as those from Adilabad for Telangana, Dharwad for Karnataka, populations of South India, Nagpur and Bharuch for Maharashtra and Gujarat states of Central India, respectively, Sringanganagar, Bhatinda and Sirsa representing Rajasthan, Punjab and Haryana of North India were used and the Mantel test for isolation-by-distance was executed using Isolation by Distance Web Service version 3.14 (1000 randomizations) (Jensen et al. 2005).

Molecular diversity and phylogenetic analysis

The phylogenetic analysis based on the maximum likelihood (ML) method was performed using MEGA6 (Tamura et al. 2013) to investigate the degree of consistency of mutation patterns in different regions. In these analyses, the nucleotide substitution for each region was selected using the Tamura–Nei model. The starting tree for ML was obtained via default neighbour-joining method, and used for the ML heuristic method with very strong branch swap filter search. The reliability of branches was assessed by 1000 bootstrap replications.

Results

Haplotype and nucleotide diversity

The length of good quality sequence of COI fragment was 487 bp. After alignment of all the sequences, no insertions or deletions were found. Alignment also revealed 24 nucleotide polymorphic sites. There were 14 parsimony informative sites and 10 singleton variable sites. Haplotype diversity and nucleotide diversity among the populations ranged from 0.561 to 0.965, and 0.00218 to 0.00719, respectively. The levels of haplotype and nucleotide diversity are found to be in ascending order for leaf hopper populations of North, Central and South India. The mean haplotype diversity (Hd) and nucleotide diversity (Pi) of COI were 0.786 and 0.00632, respectively (Table 2).

Haplotypes

Thirty haplotypes were detected in 67 samples. Haplotype ABB12 was observed to be the dominant one shared by 31 sequences represented by samples from 26 locations of three northern states of India, four locations of Central India, and one location of South India, whereas the haplotype ABB2 was shared by 3 samples from South. Out of 30 haplotypes, 25 were found to be unique haplotypes covering south (9), central (3) and North (13) Indian leaf hopper populations. Three haplotypes (ABB8, 10, and13) from south India and one haplotype (ABB16) from central India was represented by two samples (Table 3). Among the changes found in different haplotypes, the transitional changes were high in number (7 = T ↔ C, 11 = A ↔ G) while there were only 6 transversions (3 = A ↔ C, 2 = T ↔ G, 1 = G ↔ C) (Figure 2). Polymorphic sites analyzed for nucleotide position in codon, identified most changes (12) at the 3rd nucleotide position in codon followed by 8 changes at the 2nd nucleotide position and least at 1st codon position with 4 changes.

Genetic divergence

In North India, genetic divergence among RJ, HR, and PB cotton leaf hopper populations varied from 0.0% to 0.4%, 1.2%, and 1.0%, respectively. Divergence range of 0.0% to 0.8% and 1.5% was noticed for populations represented from TA and KA states of Southern India, while it was 0.0% to 1.2% and 1.5% for MH and GJ from Central India. Overall, genetic divergence for 67 samples from seven states of India showed close relationships with less than 1.9% divergence and the mean values were less than 0.7%.

Geographical structure

Genetic differentiation estimates of F_{ST} between geographically distinct populations of *A. biguttula biguttula* ranged from 0.0089 to 0.8429. Pairwise F_{ST} values between the 12 out of 21 populations comparison were found significant (Table 4).

Table 2. Haplotype and nucleotide diversity of cotton leaf hopper populations in India.

Location	No. of sequences	K	Hd	Pi	Haplotype	S
North India	39	1.062	0.561	0.00218	14	15
Rajasthan (RJ)	17	0.470	0.426	0.00097	5	4
Punjab (PB)	17	1.161	0.669	0.00239	8	9
Haryana (HR)	5	2.600	0.700	0.00534	3	6
Central India	9	3.500	0.806	0.00719	5	7
Maharashtra (MH)	4	3.833	0.833	0.00787	3	6
Gujarat (GJ)	5	3.800	0.700	0.00780	3	7
South India	19	2.924	0.965	0.00600	14	9
Telangana (TA)	15	2.895	0.961	0.00595	12	9
Karnataka (KA)	4	2.666	0.833	0.00548	3	5
All	67	3.072	0.786	0.00631	30	

Table 3. Genetic differentiation (F_{ST} values) between populations of *A. biguttula biguttula* (Ishida) from India based on mtCOI region or AMOVA haplotype F_{ST} results for pairwise population comparison.

TA	KA	MH	GJ	RJ	PB	HR
TA 0.00000						
KA 0.06640	0.00000					
MH 0.07083	0.03704	0.00000				
GJ 0.12806[a]	0.21991	−0.17414	0.00000			
RJ 0.62511[b]	0.84297 [b]	0.62534 [b]	0.39653	0.00000		
PB 0.57473 [b]	0.75556 [b]	0.50406 [a]	0.28680 [a]	0.00893	0.00000	
HR 0.49242 [b]	0.61889 [b]	0.31945 [a]	0.15789	0.21537 [a]	0.09001	0.00000

TA: Telangana; KA: Karnataka; MH: Maharashtra; GJ: Gujrat; RJ: Rajasthan; PB: Punjab; HR: Haryana.
[b]$0.001 < p < 0.01$; [a]$0.01 < p < 0.05$.

The highest pairwise F_{ST} value was found between the south and north Indian populations that were geographically divergent from one another (Table 4). Leaf hopper populations of TA and KA state of South India showed greater differentiation in pairwise comparisons with north and central India population. Comparison between KA and RJ population of geographically furthest location showed highest pairwise F_{ST} value. Populations of nearest geographical locations depicted lower pairwise F_{ST} value and least differentiation. AMOVA results showed significant molecular variations (55.09%, df = 66, $p = 0.001$) within populations which are highly influenced by inter-population differences, (44.91%; df = 6, $p = 0.001$). The COI gene flow (Nm) among different populations was variable. There was higher gene flow rate found in southern and central Indian leafhopper populations (Nm > 1) while it was low between southern and northern Indian populations (Table 5).

Mantel test revealed a significant positive correlation between the genetic and geographic distances of *A. biguttula biguttula* population collected from different locations across India (Figure 2). Based on these results, phylogeographic structure of the *A. biguttula biguttula* might be attributed to genetic isolation by distance ($r = 0.6433$; $p < 0.0010$).

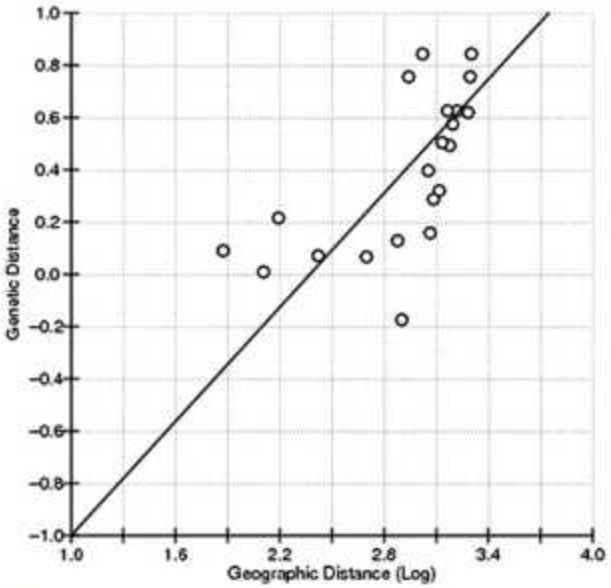


Figure 2. MANTEL TEST for matrix correlation between genetic distance and log (geographic distance): $r = 0.6433$; $p < 0.0010$.

Table 4. Gene flow (Nm) between populations of *A. biguttula biguttula* (Ishida) from India based on mtCOI region.

Population 1	Population 2	Nm	Gene flow
TA	KA	3.17	High
TA	MH	9.81	High
TA	GJ	2.52	High
TA	RJ	0.16	Low
TA	PB	0.20	Low
TA	HR	0.26	Intermediate
KA	MH	6.50	High
KA	GJ	0.84	Intermediate
KA	RJ	0.09	Low
KA	PB	0.11	Low
KA	HR	0.15	Low
MH	GJ	−1.68	Nil
MH	RJ	0.40	Intermediate
MH	PB	0.47	Intermediate
MH	HR	0.56	Intermediate
GJ	RJ	1.07	High
GJ	PB	1.28	High
GJ	HR	1.33	High
RJ	PB	27.75	High
RJ	HR	4.35	High
PB	HR	12.79	High
South India	North India	0.17	Low
Central India	North India	0.55	Intermediate
South India	Central India	1.91	High

Demographic history analysis

Tajima's *D* test, Fu and Li's *D* test and Fu and Li's *F* neutrality tests were performed to analyze demography history. Neutrality tests were rejected for North Indian population with significant negative values (Table 5). All the three neutrality test values were negative for populations of North India and significant deviations were observed between RA and PB populations of north India. Significant negative deviations from zero in populations of North India indicate that there is an excess of rare mutations favouring population expansion or growth. These results reject the hypothesis of neutral evolution for cotton leaf hopper population from North India. The central and south Indian leafhopper populations with positive values showed the sign of bias towards intermediate frequency but were non-significant. Non-significant negative values were observed for population of KA (South India).

Phylogenetic analysis

The phylogenetic tree for COI haplotype was constructed using *Empoasca vitis* as an outgroup. The phylogenetic tree for COI gene resulted in two distinct haplotype clusters, one clade represented by leafhopper populations of North India, with populations of South India in another clade. Leafhopper populations from Central India were distributed in both the

Table 5. Tajima's *D* test, Fu and Li's *D* test and Fu and Li's *F* for COI gene in populations of leaf hoppers.

Location	Tajima's *D*	Significance	Fu and Li's *D*	Significance	Fu and Li's *F*	Significance
North	−2.32418	$^{**}p < 0.01$	−2.89923	$^{*}p < 0.05$	−3.19698	$^{*}p < 0.05$
Central	1.66112	NS, $p > 0.10$	0.92083	NS, $p > 0.10$	1.20849	NS, $p > 0.10$
South	0.47277	NS, $p > 0.10$	0.36472	NS, $p > 0.10$	0.45706	NS, $p > 0.10$
Rajasthan (RJ)	−1.84308	$^{*}p < 0.05$	−2.46944	$^{*}p < 0.05$	−2.63848	$^{*}p < 0.05$
Punjab (PB)	−2.03151	$^{*}p < 0.05$	−2.53258	$^{*}p < 0.05$	−2.75714	$^{*}p < 0.05$
Haryana (HR)	−0.66823	NS, $p > 0.10$	−0.66823	NS, $p > 0.10$	−0.69243	NS, $p > 0.10$
Gujarat (GJ)	0.91278	NS, $p > 0.10$	0.91278	NS, $p > 0.10$	0.95142	NS, $p > 0.10$
Maharashtra (MH)	1.66214	NS, $p > 0.10$	1.66214	NS, $p > 0.10$	1.59765	NS, $p > 0.10$
Telangana (TA)	0.17251	NS, $p > 0.10$	0.45322	NS, $p > 0.10$	0.43255	NS, $p > 0.10$
Karnataka (KA)	−0.21249	NS, $p > 0.10$	−0.21249	NS, $p > 0.10$	−0.20080	NS, $p > 0.10$
All	−1.29609	NS, $p > 0.10$	−1.93502	NS, $0.10 > p > 0.05$	−2.02823	NS, $0.10 > p > 0.05$

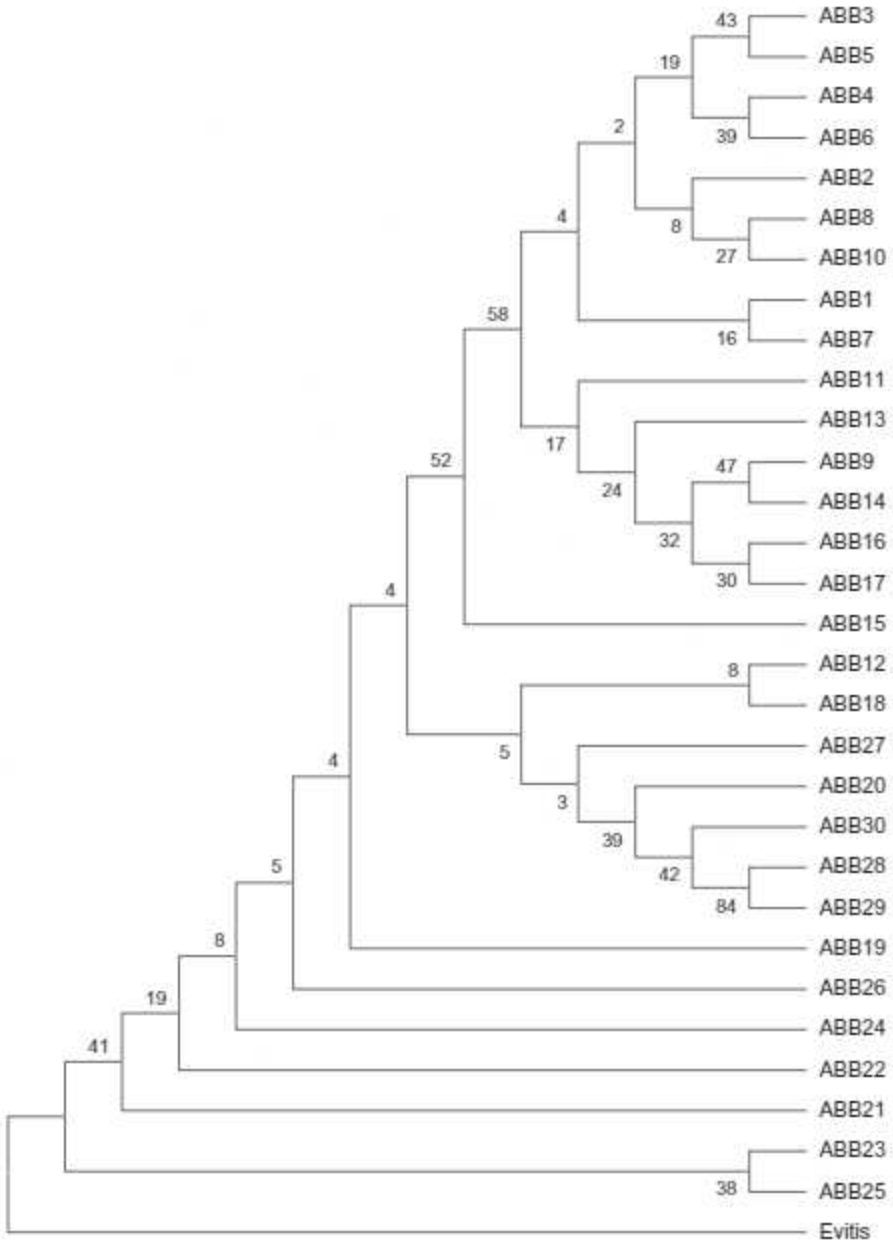


Figure 3. Phylogenetic tree of the 30 mtCOI DNA haplotypes in the *A. biguttulla biguttulla*.

clades. Most haplotypes were weakly associated (less than 50% bootstrap support) while nodes connecting branches of main clusters (52%) obtained marginal support. Haplotype group ABB28/ABB29 (84%) was the lone clade that showed strong association with well-supported bootstrap values (Figure 3).

Discussion

The cotton leaf hopper is a key pest of cotton in Indian subcontinent. It was reported as serious pest of cotton in 1918 from Nagpur India. The occurrence of this pest was recorded from various cotton-growing states of India (Fletcher 1920). Earlier nomenclature described it as *Empoasca devastans* Distant. More than two dozen of leaf hopper species belonging to the genus *Empoasca* are reported to occur in India. The genus *Empoasca* has been renamed as *Amrasca* by Ghauri (1967). Dworakowska (1970) described the genus as *Sundapteryx* and then as *Sundapteryx biguttula biguttula* (Ishida). Kapoor and Sohi (1972) renamed it as *A. biguttula bigutula* (Ishida). It is also known by other names such as *Amrasca devastans* (Dist.). and *A. biguttula* (Ishida). Based on the morphological observations, the species occurring on cotton across India was reported as single. But, studies to validate the single species nature of *A. biguttula biguttula* spread across India have not been reported so far.

In the present study, COI-based mean genetic divergence from 67 samples of leaf hoppers representing seven cotton-growing states spanning 2000 km of geographical area was found to be less than 0.7%. The value falls within the limit of the 2% genetic distance for insect species boundary (deWaard et al. 2011; Fu et al. 2014), confirming the single predominant species occurrence on cotton. The results are in concurrence and comparable with similar studies on plant

leafhoppers where intraspecific genetic variation of COI sequence within and among different geographical locations was reported to be 0–0.23% and 0.12% for *Nilaparvata lugens* and *Sogatella furcifera* (Hemiptera: Delphacidae), respectively. Smaller genetic distances of 0.3–1.2%, based on COI, 16SrDNA and combined sequences among the populations of *E. vitis, J. formosana,* and *E. onukii* from Mainland China, Taiwan, and Japan provided evidence towards the hypothesis of single species (Fu et al. 2014).

Among the 30 haplotypes identified, haplotype ABB12 was found to be dominant comprising of 31 samples with major representation of populations from North India. This suggests that the North Indian population of leafhoppers belong to one dominant genotype. Variation in the COI gene sequence showed codon position bias. It has been observed that the third nucleotide position shows highest variation while the first base of codon is most stable (Hebert et al. 2003; Castanhole et al. 2013). Nucleotide variation might be due to transitions, transversions, indels, additions or deletions. COI sequence variation of the populations depicted only transitions and transversions. The number of transitions outnumbered the transversions and is in concurrence with the evidence that transitional bias or more frequent transitions are observed during the comparison of closely related taxa (Brown et al. 1982; Arias & Sheppard 2005).

Comparison of seven geographically distinct population groups revealed a significant pair-wise genetic differentiation (F_{ST}) values between the 12 out of 21 populations. The highest pairwise F_{ST} values were found between populations collected from locations that were furthest from each other. North Indian population group is geographically well-separated from central and south Indian populations. Higher gene flow (Nm) value was noticed between the population groups in closer vicinity and contributes to least genetic differentiation. The genetic differentiation and gene flow analysis together with strong positive correlations of F_{ST} values with geographic distance from the Mantel test confirms the phylogeographic structure of the *A. biguttula biguttula* Ishida as isolated by distance. Positive correlation of pairwise estimates of F_{ST} for mtCOI sequences of 23 populations of *Graphocephala atropunctata* to the geographical distance, endorsed phylogeographic structure to isolation by distance (Ballman et al. 2011)

Significant negative deviations from zero in the three neutrality tests such as Tajima's *D* and Fu and Li's *D* and *F* test rejected the hypothesis of neutral evolution for leaf hopper populations from North India and support population expansion. The South and Central India populations do not show such pattern. Phylogenetic tree also supports the geographic separation of populations. The tree shows two different clades of North and South India with the central Indian population distributed in both clades.

Population structure of insect species is largely affected by individual or combinations of environmental and ecological factors, natural barriers, alternative host plants, migration, and human activities (Fairley et al. 2000; Duan et al. 2013). Insecticide application exerts a strong selection pressure on insect populations. The intensity of insecticide application varies in different regions of India. The cotton leafhoppers from north India are considerably more susceptible to neonicotinoids as compared to leafhopper populations from Central or South India (Kranthi et al. unpublished). The higher resistance for the neonicotinoids in central and south India clearly demonstrated variability in the geographic populations of leafhoppers. The observed variability could also be a result of variation in factors such as cropping patterns, availability of alternate host plants, insecticide use patterns, cultivation of Bt hybrids of varying susceptibility and possible genetic variability, and different climatic conditions that act individually or in combination on the target insect.

The genetic diversity analysis study will help to track dynamic changes occurring in the population and thereby will assist in the process of designing efficient control strategy. The phylogenetic tree for COI gene resulted in two distinct haplotype clusters of population groups of North and South India. But, most haplotypes were weakly associated (less than 50% bootstrap support). A similar result was reported by Kim et al. (2000) and Li et al. (2006), wherein most nodes were very weakly supported or unresolved, indicating close phylogenetic relationships among diamond backed moth, *Plutella xylostella* haplotypes. Further, the population-based analysis also indicated that many *P. xylostella* populations are genetically similar to each other, with a high gene flow rate.

We found that North Indian leaf hopper population is significantly different with respect to partial COI sequences from Central and South Indian leaf hopper populations. The genetic differentiation and gene flow analysis together confirmed the phylo-geographic structure of the *A. biguttula biguttula* Ishida as isolated by distance.

Disclosure statement

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