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Genetic variability and population structure of *Leucinodes orbonalis* (Guenée), a severe insect pest of brinjal in India

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Abstract

Aim: The present study was undertaken to provide valuable insights regarding population genetic structure of *Leucinodes orbonalis* from diverse agro-ecologies of India.

Methodology: Molecular characterization of *L. orbonalis* populations collected from five major agro-climatic zones of India was carried out using mitochondrial cytochrome oxidase I (COI) gene. Collected specimens were subjected to DNA extractions, partial PCR amplification and sequencing of the target gene, and multiple sequence alignments.

Results: The results showed very less diversity in the nucleotide positions of the COI sequences of 79 studied specimens, with a low number of segregating sites (30), nucleotide diversity (0.00438) and overall mean genetic distance (0.004 ± 0.001). The significant negative values of neutrality tests and unimodal mismatch distribution supported the demographic expansion theory in Indian *L. orbonalis*. Analysis of the molecular variance revealed that 93.13% of the genetic variation was within populations, and the variation among populations was only 6.87%. The pairwise genetic differentiation was also found to be low to moderate between most of the populations. Multiple haplotypes were recorded from all the populations, and both neighbour-joining tree as well as the haplotype network showed that clustering of the haplotypes was independent of the geographical location.

Interpretation: Thus, it can be inferred that Indian populations of *L. orbonalis* have very low genetic variation levels concerning the COI gene. There is a possible occurrence of stable inherited gene flow among populations, thereby reducing genetic variation in India.

Key words: Brinjal, Diversity, *Leucinodes orbonalis*, Mitochondrial COI gene, Phylogeny

L. orbonalis moth

Gel documentation of amplified COI gene

Nucleotide sequencing

Homogenous genetic structure of *L. orbonalis* in India

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Introduction

Brinjal (*Solanum melongena* L.), also known as aubergine or eggplant, is a highly productive and popular vegetable crop cultivated throughout the warmer regions of the world. Being a rich and cheap source of many essential minerals and vitamins, consumption of brinjal quickly helps to meet the vital nutrient requirements even in malnourished people (Palraju *et al.*, 2018), and is considered as “the poor man's vegetable”. In India, it is considered as a cash crop mostly by small and marginal farmers, and is cultivated in an area of about 730 ha with annual production of 12801 metric tonnes, which is about 9 percent of the total vegetable production in the country (Saikia *et al.*, 2021). Though hardy for abiotic stress, this crop is prone to attack from several insect pests and diseases.

Among these, shoot and fruit borer, *Leucinodes orbonalis* Guenée (Lepidoptera: Crambidae) is considered to be the most serious and destructive one, and has become the major production constraint in several brinjal growing countries across tropical and subtropical Africa and Asia, including India (Karthika *et al.*, 2019). In India, it has been rated as a key pest of brinjal in inflicting an average annual yield loss ranging up to 70-80 percent (Tripura *et al.*, 2017), in the absence of proper control measures. Recently, this pest species has also been included in the list of quarantine pests of “A-1 category” for many countries outside its reported native range (EPPO, 2020). Farmers predominantly apply synthetic contact insecticides to control this offensive pest. However, *L. orbonalis* larvae usually escape insecticidal sprays because of its concealing nature within shoots and fruits (Shelton, 2010), thereby tending desperate farmers to overspray insecticides. Nevertheless, such indiscriminate use of synthetic chemicals has been found to induce several ill effects on environment mainly non-target organisms along with high pesticide residues on consumable fruits rendering severe threat to human health. Further, the continuous stress induced by insecticides application has made the *L. orbonalis* populations adapt themselves from these ravages primarily through changing their form of feeding physiology, reproduction and eco-behavioural pattern (Palraju *et al.*, 2018) or via genetic variations in their population (Karthika *et al.*, 2019).

The genetic plasticity of this pest species is quite apparent in its ability to rapidly detoxify several insecticides in addition to secondary metabolites present within its limited host range (Ranjithkumar *et al.*, 2013). Certain investigations have even hypothesized that apart from this species, there are several other species in the genus *Leucinodes*, conspecific to *L. orbonalis* (Mally *et al.*, 2015) and South Asia particularly India, is the most probable centre of origin for the genus *Leucinodes* (Gilligan and Passoa, 2014), thereby hinting about the existence of unidentified cryptic species complex in the region. The variable response of *L. orbonalis* adults to pheromone trap catches in different regions of India has further elicited the doubt regarding possible variation in the population genetic structure of *L. orbonalis* in the country (Shashank *et al.*, 2015).

Detailed knowledge regarding genetic variations existing among insect pest populations along with understanding the mechanisms that drive genetic changes is prerequisite for devising long term pest management strategies (Murali *et al.*, 2017). Study of pest population structure is also a more accurate means to determine phenotypic and genetic changes they undertake to tackle various survival hurdles (Sambathkumar *et al.*, 2017). Molecular markers are considered as essential tools for assessment of genetic variability at the population level. Among these, mitochondrial genes have the edge of being present in a high number of copies in each cell, enabling their fast retrieval and amplification (Gissi *et al.*, 2008). Further, characteristics like maternal inheritance with negligible recombination, conserved composition and lack of introns, make them a more suitable choice for evolutionary studies than nuclear gene markers (Filipova *et al.*, 2011).

The availability of a wide range of primers for amplification of the mitochondrial cytochrome oxidase I (COI) gene from diverse invertebrate and vertebrate phyla establish this gene as a good target for species identification in animals (Atikah *et al.*, 2020). The COI gene has also been found to be useful in differentiating cryptic species (Shashank *et al.*, 2018), as well as for the assessment of intraspecific diversity in insects (Chatterjee *et al.*, 2019), due to its large size and high nucleotide substitution rate (Mandal *et al.*, 2014). Thus, the present study was undertaken to precisely trace the genetic variability among different geographic populations of *L. orbonalis* from India based on cytochrome oxidase I (COI) gene. Such studies will pave way for understanding physiological or behavioural changes, and population dynamics and damage thresholds in different ecological regions, for designing better management strategies against *L. orbonalis*.

Materials and Methods

Specimen collection and DNA isolation: *L. orbonalis* populations were collected from different localities of five major agro-climatic zones of India, i.e., Middle Gangetic Plain (Varanasi; 30°54' N, 75°48' E, 80.71 m above mean sea level), Trans Gangetic Plain (Delhi; 29°10' N, 75°42' E, 224 m above mean sea level), Eastern Plateau and Hills (Raipur; 28°64' N, 77°16' E, 298.15 m above mean sea level), Western Plateau and Hills (Pune; 29°01' N, 79°48' E, 560 m above mean sea level), and Southern Plateau and Hills (Bengaluru; 24°34' N, 73°42' E, 920 m above mean sea level), between February 2015 and June 2017. At each collection site, about 50 larvae were collected from infested brinjal fruits, and these larvae were reared and allowed to pupate under laboratory conditions (Biocontrol Laboratory, Department of Entomology and Agricultural Zoology, Banaras Hindu University) at a temperature of 27 ± 2°C and relative humidity of 80 ± 5 % on the potato as an alternative diet.

The emerged adults were used for DNA extraction. The collected specimens were also reconfirmed as *L. orbonalis* based on structure of male and female genitalia, using the keys provided

by Mally *et al.* (2015). DNA was isolated by the method of Murray and Thompson (1980) with some modifications, using cationic detergent Cetyl Trimethyl Ammonium Bromide (CTAB). The quantification of DNA samples was conducted by UV spectrophotometry (Elico® SL191), and the quality was checked on 0.8% agarose gel.

Amplification and sequencing of COI gene fragment: The polymerase chain reactions were carried out on DNA extracts to amplify the partial sequence of COI gene using the primers Lep-F1 (forward) and Lep-R1 (reverse), following the protocol of Kress and Erickson (2012). The master mix (25µl) contained 2µl DNA template (100ng), 2µl PCR buffer (10X), 0.5µl Taq polymerase (1U, GeNei™), 1µl each of dNTP (2mM), MgCl₂ (2mM) and specific primers (10pmol), and 17.5µl of nuclease-free water. The amplification was performed in a thermocycler (BIO-RAD MJ Mini™) programmed at: initial preheating for 5 min at 94°C; followed by 30 cycles of amplification, each cycle with the following profile: denaturation for 30 sec at 94°C, annealing for 45 sec at 50°C and extension for 1 min at 72°C. This was followed by a final extension for 10 min at 72°C. The amplified products were examined on 1% agarose gel, purified (GeneJET Gel Extraction Kit, Thermo Scientific) and then sequenced using Sanger's method in ABI 3730 automated DNA analyzer at SciGenom Labs Pvt Ltd. (Cochin, India).

Sequence data analyses: The COI sequences were imported into FASTA format for alignment and trimming in ClustalW algorithm by setting default parameters in the MEGA 6.0 (Tamura *et al.*, 2013) software application package. Sequences were analyzed based on homologs of the gene when subjected to nBLAST of National Center for Biotechnology Information (NCBI) and then submitted for obtaining GenBank accessions. The diversity indices such as number of segregating sites, number and diversity of haplotypes, nucleotide diversity were determined using the software package DnaSP 5.10.1 (Librado and Rozas, 2009). Further, to ascertain demographic expansion, neutrality tests like Tajima's D, Fu's F and mismatch distribution analysis were also performed with DnaSP 5.10.1. The average nucleotide base composition of COI sequences and overall mean genetic distance were calculated by MEGA 6.0. For understanding the genetic structure, analysis of molecular variance (AMOVA) and

pairwise Fst values were computed using Arlequin 3.5 (Excoffier and Lischer, 2010). Level of significance was determined with 1000 permutation replicates. The haplotype network was created using a median-joining algorithm in Network 4.6.1.1 software (Bandelt *et al.*, 1999), while the haplotype based neighbour-joining tree was constructed using MEGA6.0 software.

Results and Discussion

In the present investigation, 630-bp fragments of the COI gene generated from 79 *L. orbonalis* specimens were used. The average nucleotide composition of partial COI gene fragments in five populations is shown in Table 1. The total mean nucleotide composition was found to be 31.60%, 14.44%, 39.52% and 14.44% for A, G, T and C. The average AT and GC content was 71.12% and 28.88%, respectively. A comparatively higher AT content is a predominant attribute of COI gene in arthropods (Shashank *et al.*, 2018). The number of haplotypes for the studied populations ranged from 2 (Bengaluru) to 12 (Raipur and Pune) (Table 2), while a total of 30 haplotypes was recorded when all the populations were taken as a whole. The Pune population had the highest haplotype diversity (0.934) and Bengaluru had the lowest haplotype diversity (0.667). The Bengaluru population also showed the lowest nucleotide diversity values (0.00317) and the Raipur population exhibited the highest nucleotide diversity (0.00560).

The overall population was characterized by low nucleotide diversity (0.00438) and high haplotype diversity (0.878). Several polymorphic sites were also observed in the COI gene sequences of studied populations, showing both transitional and transversional substitutions. Tajima's D and Fu's F neutrality tests were performed to evaluate demographic history in Indian *L. orbonalis*. For most populations, neutrality tests were rejected as they had significant negative values (Table 2). This endows the hypothesis that population expansion events took place in the past. Such negative neutrality test values for all the studied populations from varied agro-climatic zones of India also indicated the occurrence of an excess of rare mutations, thereby favouring population growth or expansion. The pooled population also had significant negative values in Tajima's D (-1.8533, $P < 0.05$) and Fu's F (-23.0520, $P < 0.01$) tests. This result was further supported by the smooth and unimodal mismatch distribution plot

Table 1: Average nucleotide base composition of COI gene sequences for different *L. orbonalis* population

<i>L. orbonalis</i> population	Nucleotide composition (Relative values)					
	Adenine (%)	Guanine (%)	Thymine (%)	Cytosine (%)	A + T (%)	G + C (%)
Varanasi	31.55	14.48	39.48	14.49	71.03	28.97
Delhi	31.63	14.42	39.53	14.42	71.16	28.84
Raipur	31.61	14.44	39.51	14.44	71.12	28.88
Pune	31.60	14.43	39.47	14.50	71.07	28.93
Bengaluru	31.59	14.44	39.63	14.34	71.22	28.78
Pooled	31.60	14.44	39.52	14.44	71.12	28.88

Here "Pooled" denotes combined set of populations from all the studied locations

Table 2: Diversity indices and neutrality test (Fu's F_s and Tajima's D) values for different *L. orbonalis* populations based on COI gene sequences

Index	<i>L. orbonalis</i> population					
	Varanasi	Delhi	Raipur	Pune	Bengaluru	Pooled
n	23	19	17	17	3	79
Hn	10	8	12	12	2	30
Hd	0.826	0.766	0.941	0.934	0.667	0.878
π	0.00348	0.00347	0.00560	0.00448	0.00317	0.00438
S	17	15	16	14	3	30
Ts	15	14	13	12	3	24
Tv	2	1	3	2	0	6
Fu's F_s	-3.6012*	-2.0231 ^{NS}	-5.4805**	-6.7955**	-1.6094 ^{NS}	-23.0520**
Tajima's D	-1.8988*	-1.8335*	-0.9857 ^{NS}	-1.2161 ^{NS}	-0.1472 ^{NS}	-1.8533*

n: number of sequences, Hn: number of haplotypes, Hd: haplotype diversity, π : nucleotide diversity, S: number of segregating sites, Ts: number of sites with transitions, Tv: number of sites with transversions. *Significant at $P < 0.05$, **Significant at $P < 0.01$, NS: Non-significant ($P > 0.10$). Here "Pooled" denotes combined set of populations from all the studied locations

Table 3: Analysis of molecular variance (AMOVA) for the COI sequences of different *L. orbonalis* populations

Sources of variation	df	Sum of squares	Variance components	Percentage of variation
Among populations	4	11.026	0.09625 Va	6.87
Within populations	74	96.595	1.30533 Vb	93.13
Total	78	107.620	1.40158	100
Fixation index, F_{ST} : 0.06494*				

*Significant at $P < 0.05$; df: Degree of freedom, Va: Variance components due to differences among the populations within the group, Vb: Variance components due to differences within the populations

Table 4: Pairwise genetic differentiation (F_{ST}) values between different populations of *L. orbonalis*

Population	Varanasi	Delhi	Raipur	Pune	Bengaluru
Varanasi	0.00000				
Delhi	0.02909 ^{NS}	0.00000			
Raipur	0.05530*	0.03412 ^{NS}	0.00000		
Pune	0.05959*	0.05576*	0.03165 ^{NS}	0.00000	
Bengaluru	0.30063**	0.22115*	0.20322 ^{NS}	0.27524*	0.00000

*Significant at $P < 0.05$, **Significant at $P < 0.01$, NS: Non-significant ($P > 0.10$)

that included combined population sets from all the studied locations. The present findings are in close agreement with recent similar studies on certain lepidopteran pests from India such as *Tuta absoluta* (Shashank et al., 2018), *Maruca vitrata* (Chatterjee et al., 2019) and *Pectinophora gossypiella* (Naik et al., 2020), including *L. orbonalis* (Palraju et al., 2018, Karthika et al., 2019).

In all these reports, neutrality tests were rejected and unimodal pairwise nucleotide distribution profiles were obtained, suggesting that all these pest populations might have undergone a sudden demographic expansion in India. The AMOVA test (Table 3) showed that the majority of total molecular variance (93.13%) was distributed within populations, and only 6.87% was partitioned among the populations. The genetic differentiation

(F_{ST}) index between groups was 0.06494 with $P < 0.05$, thereby indicating that the genetic differentiation of the population was significant. Further, the pairwise F_{ST} values ranged between 0.00 and 0.30, with an average of 0.1345. Of the total 14 comparisons, 5 showed significant genetic differentiation. Following the criterion given by Wright (1978), genetic differentiation was considered low for F_{ST} values less than 0.05, moderate for values between 0.05 and 0.15, high for values between 0.15 and 0.25, and very high for values exceeding 0.25.

The values of pairwise F_{ST} for all the populations were low to moderate, except in Bengaluru, which showed very high genetic differentiation from others (Table 4). The overall mean genetic distance was also found to be very low (0.004 ± 0.001),

Fig. 1: Neighbour-joining phylogenetic tree of 30 mitochondrial COI gene haplotypes of Indian *L. orbonalis*.

and this strongly supports single species status of *L. orbonalis* in the country, as a divergence of more than five percent in the COI gene amplicon, *i.e.*, a genetic distance exceeding 0.05, depicts the likely occurrence of a new species in *Lepidoptera* (Hebert *et al.*, 2010). Shashank *et al.* (2015) also reported that pairwise genetic distance among nine different geographic populations of *L. orbonalis* from India for the COI gene ranged between 0.000 and 0.016, thereby suggesting the existence of not much genetic variations in this pest species. However, few high genetic differentiation values indicate the emergence of specific habitat

requirement based genetically distinct populations in Indian *L. orbonalis* as also suggested by Murali *et al.* (2017) and Palraju *et al.* (2018). All the populations also displayed a good number of mitochondrial haplotypes for the COI gene. Of the 30 haplotypes, 17 haplotypes were unique, and the remaining was shared at least by two populations. In the haplotype network, Haplotype 1 was most common, and it was shared by all the populations. This suggested that Haplotype 1 might be the ancestral haplotype, as it had an internal position in the network and at high frequencies, several lineages arose from it. Few unique haplotypes that exist

independently in specific geographical populations show a certain degree of genetic differentiation, as revealed by Xu *et al.* (2019), for *Calliptamus italicus* populations of Sino-Kazakh region based on mitochondrial gene markers (COI and COII). The neighbour-joining phylogenetic tree was shallow, and there were no significant clusters or genealogical branches of samples, corresponding with their respective sampling sites (Fig. 1).

This was also consistent with the observation that a population expansion event might have occurred after a bottleneck in the past, as suggested by Zheng *et al.* (2019). These further indicate about the existence of very low genetic diversity among studied populations. Earlier studies regarding genetic diversity analyses of Indian *L. orbonalis* populations based on RAPD markers (Geetharajalakshmi *et al.*, 2006, Marimuthu *et al.*, 2009) as well as COI gene (Shashank *et al.*, 2015, Palraju *et al.*, 2018, Karthika *et al.*, 2019) also strongly support the present findings, as they also hinted about existence of genetic homogeneity among geographically isolated populations of *L. orbonalis* in India. However, all these earlier studies were mainly confined to southern states of the country. There can be two probable reasons for very low genetic variations among Indian populations of *L. orbonalis*. Firstly, it is well documented that several lepidopteran moths have high migratory potential.

Thus, due to substantial gene flow, many of them often exhibit low genetic differentiation over their wide geographical distributional ranges (Sun *et al.*, 2015). In case of *L. orbonalis*, both the active migration (because of good flight capacity) and passive migration (because of long distance transport of infested brinjal fruits) occurs, which might be helping it to maintain a steady state of gene flow (Murali *et al.*, 2017), thereby minimizing genetic variation. The second reason can be that *L. orbonalis* has a narrow host range, and most of the commercially grown varieties of brinjal show little variation amongst themselves for their susceptibility against this pest species (Marimuthu *et al.*, 2009). Thus, there is no selection pressure exerted on this pest due to host plants, preventing it from undergoing any genetic changes unlike certain other lepidopterans, mostly from Noctuidae (Subramanian and Mohankumar, 2006) and Tortricidae (Emelianov *et al.*, 1995) families, where the existence of host-associated genetic differences are widespread.

In conclusion, this study provides a clear image regarding the homogenous genetic structure of *L. orbonalis* in the country, possibly due to the occurrence of stable inherited gene flow among populations. Such information will be very helpful in designing sustainable management strategies for this pest species in an area-wide manner across India.

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Add-on Information

Authors' contribution: K.G. Padwal: Collected the insect samples, carried out the experiments, and prepared the first draft of the manuscript; S. Chakravarty: Performed data analysis and interpretation, and edited the manuscript draft and C.P. Srivastava: Conceptualized the idea, planned the methodology, supervised the experiments, and wrote, evaluated and finalized the manuscript draft.

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